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The role of serine protease kallikrein 5 in skin barrier dysfunction—a potential therapeutic intervention for atopic dermatitis

Yanan Zhu

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Department of Dermatology

Institute of Child Health

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Declaration

I, Yanan Zhu, confirm that all the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Abstract

Atopic dermatitis (AD) is a common, chronic inflammatory skin disorder caused by both genetic and environmental factors. The primary cause of AD is epidermal barrier dysfunction, which leads to impaired protective function of skin and promotes Th2 immune response. The barrier defect in AD can be induced by dysregulations of numerous molecules, including imbalance between serine proteases kallikreins (KLKs) and their inhibitor LEKTI. The importance of serine protease-protease inhibitor balance has also been revealed in Netherton Syndrome (NS), which is caused by loss-of-function mutations in the LEKTI encoding gene *SPINK5*. Hyperactivity of KLKs in NS can result in severe skin barrier defect. AD and NS both exhibit up-regulated activity of KLKs, and they share some clinical features including atopy and skin inflammation. Therefore, it was speculated that up-regulated KLKs might play an important role in AD pathogenesis.

In this project, up-regulation of a key epidermal protease kallikrein 5 (KLK5) and disturbed expression of barrier-related proteins were confirmed in AD skin. In order to eliminate the influences of other causes of AD and investigate the role of up-regulated KLK5 in barrier defect, keratinocytes overexpressing KLK5 (KLK5-cells) were generated. Over-degradation of desmoglein 1 (DSG1) and impaired function of protease activated receptor 2 (PAR2) were found in KLK5-cells. *In vitro* organotypic cultures and *in vivo* skin grafts generated from KLK5-cells exhibited AD-like histological features and barrier abnormalities including disrupted keratinocyte growth. In order to further study the influences of up-regulated KLK5 on keratinocyte growth and cytokine production, related phospho-kinase and cytokine arrays were performed. There were increased levels of p53, heat shock protein 60 (HSP60) and elevated secretion of IL-8, thymic stromal lymphopoietin (TSLP) and IL-10 in KLK5-cells. As p53 and HSP60 are involved in the regulation of cell growth and cytokine production respectively, up-regulation of KLK5 could result in disorganized keratinocyte proliferation/differentiation and aberrant cytokine levels by up-regulating p53 and HSP60, consequently exacerbating the skin barrier dysfunction in AD. Finally, sunflower trypsin inhibitor analogue (SFTI-G) was used to inhibit the unopposed activity of KLK5. The results showed restored level of DSG1, reduced expression of p53/HSP60 and decreased production of inflammatory cytokines in KLK5-cells treated with SFTI-G. Therefore, improvement of epidermal barrier function by inhibiting up-regulated KLK5 is a promising therapeutic intervention for AD.

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Abbreviations

AD	Atopic dermatitis
AMPs	Antimicrobial peptides
AOI	Area of interest
b-defensins	Beta-defensins
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDSN	Corneodesmosin
CE	Cornified envelope
CSTA	Cystatin A
DAPI	4',6-diamidino-2-phenylindole
DED	De-epidermalized dermal matrix
Der-f	Dermatophagoides farinae
Der-p	Dermatophagoides pteronyssinus
DMEM	Dulbecco's Modified Eagle Medium
DSC1	Desmocollin 1
DSG1	Desmoglein 1
EDC	Epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELA2	Elastase-2
ELISA	Enzyme linked immunosorbent assay

Erbin	Erbb2 interacting protein
FCS	Fetal calf serum
FLG	Filaggrin
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GSK3β	Glycogen synthase kinase 3 beta
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60
H&E staining	Haematoxylin and eosin staining
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL-8	Interleukin-8
IL-10	Interleukin-10
KLK	Kallikrein/Kallikrein-related peptidase
KLK5	Kallikrein 5
KLK7	Kallikrein 7
MMPs	Matrix metalloproteases
MOI	Multiplicity of infection
NFkB	Nuclear factor kappa B
NMFs	Natural moisturizing factors
NS	Netherton Syndrome
LB	Lamellar body
LEKTI	Lympho-epithelial Kazal-type inhibitor
LEKTI-2	Lympho-epithelial Kazal-type inhibitor 2

LEKTI-3	Lympho-epithelial Kazal-type inhibitor 3
PAGE	Polyacrylamide gel electrophoresis
PARs	Protease-activated receptors
PAR2	Protease-activated receptor 2
PAR2-AP	PAR2 activating peptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Pen/Strep	Penicillin/streptomycin
OTC	Organotypic culture
RFU	Relative fluorescence unit
rKLK5	Recombinant Kallikrein 5
RT-PCR	Reverse transcription polymerase chain reaction
S. aureus	Staphylococcus aureus
SB	Stratum basale
SC	Stratum corneum
SCCE	Stratum corneum chymotryptic enzyme
SCTE	Stratum corneum tryptic enzyme
SDS	Sodium dodecyl sulphate
Serpin	Serine protease inhibitor
SFFV	Spleen focus-forming virus promoter
SFTI-1	Sunflower trypsin inhibitor
SG	Stratum granulosum
SPI	Serine protease inhibitor

SPINK5	Serine protease inhibitor Kazal-type 5
SPINK6	Serine protease inhibitor Kazal-type 6
SPINK9	Serine protease inhibitor Kazal-type 9
SS	Stratum spinosum
TEWL	Transepidermal water loss
TSLP	Thymic stromal lymphopoietin
TU	Transducing units
UV	Ultraviolet

CHAPTER 1: INTRODUCTION

1.1 The epidermal barrier

Skin is composed of dermis and epidermis. It is the largest organ in human body and serves as a physical and chemical barrier against the external environment. Skin can protect the internal tissues from mechanical stress, UV radiation and harmful agents. Other main functions of skin include sensation, thermoregulation and prevention of body fluid loss. The role as 'first line of defence' of skin is attributed to the epidermal barrier, which resides within the epidermis, the outermost layer of skin.

1.1.1 The epidermis

The epidermis is a highly stratified epithelium, which mainly consists of stratum basale (SB, basal layer), stratum spinosum (SS, spinous layer), stratum granulosum (SG, granular layer) and stratum corneum (SC, cornified layer) (Figure 1.1).

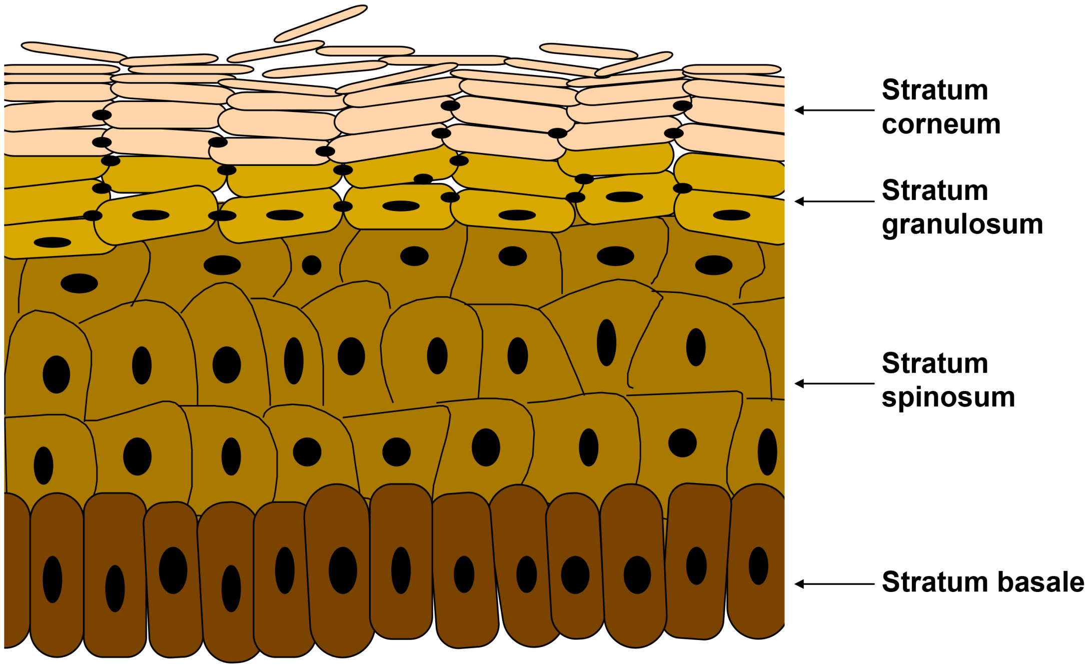

Figure 1.1. Structure and composition of the epidermis.
The epidermis is mainly composed of stratum basale, stratum spinosum, stratum granulosum and stratum corneum.

The keratinocyte is the predominant cell type in the epidermis. Each keratinocyte migrates superficially from the SB to the SC. They undergo cell proliferation, differentiation and desquamation along the way with complete cell turnover occurring every four weeks. Therefore, the epidermis is a dynamic tissue, which is continuously renewed through proliferation of keratinocyte stem cells in the SB.

Proliferated keratinocytes move upward to the suprabasal layers and commit to differentiation. In the upper SS and SG, lamellar granules appear in the cells. These granules can release numerous types of lipids and proteins, which are secreted into the extracellular space during the formation of SC. For instance, lamellar granules deliver not only lipid constituents/lipid precursors but also the enzymes required to form the lipid matrix in the SC, which encases the corneocytes like mortar and prevents internal water loss. The proteases and protease inhibitors released by lamellar bodies orchestrate the orderly degradation of adhesion molecules corneodesmosomes, further allowing corneocytes to dissociate and shed from the skin surface (Caubet et al. 2004; Brattsand et al. 2005). In addition, antimicrobial peptides (AMPs) secreted by the lamellar granules play important roles in innate immunity. They are able to protect the skin against infectious agents (Oren et al. 2003; Braff et al. 2005). As cells continuously migrate upwards, the appearance of keratinocytes changes dramatically. The nuclei and cytoplasma start to disappear and the keratin intermediate filaments aggregate to form microfibrils, and the cells start to transform into the corneocytes that form the SC.

During the final stage of terminal differentiation, keratinocytes change from metabolically active cells to corneocytes. Corneocytes are flattened cells, which lost their nuclei and sub-cellular organelles and become densely packed with keratin fibres. In order to maintain certain thickness of the epidermis, corneocytes are separated from each other through the proteolysis of intercellular adhesion molecules and shed from the skin surface. This process is known as desquamation. The corneocytes that flake off from the epidermis are continually replaced by the underneath keratinocytes undergoing terminal differentiation (Rook and Burns 2004).

Thus, various layers work in concert to provide strength and flexibility of the epidermis. The growth of keratinocyte is modulated by multiple pathways, which involve the functions of various molecules. For instance, transforming growth factor-mediated signalling could control keratinocyte proliferation and differentiation (Dahler et al. 2001). Mitogen-activated protein kinases also play a regulatory role in keratinocyte differentiation (Eckert et al. 2002). Transcription factor p53 may participate in the modulation of keratinocyte proliferation and differentiation (Dazard et al. 2000). In addition, the process of epidermal desquamation is strictly regulated by proteases and protease inhibitors. Therefore, the rates of keratinocytes proliferation, differentiation and desquamation are delicately balanced to allow a continual renewal of the epidermis and leave the skin sufficiently intact to exert its defensive function.

1.1.2 The epidermal barrier

The protective function of skin relies on epidermal barrier, which is established during the formation of SC. When granular keratinocytes transform into corneocytes, the plasma membrane is replaced with an insoluble protein layer called 'cornified envelope' (CE). Meanwhile, the lamellar granules secret numerous types of lipids into the extracellular space at the interface between SG and SC. These lipids are orderly organized to form the lamellar membranes. One layer of lipids binds to cornified envelope covalently, and multiple layers of these membrane structures fill the spaces between the corneocytes. Furthermore, the structural integrity of the SC is also maintained by specialized desmosomes called corneodesmosomes, which bind the corneocytes together by incorporating into the cornified envelope.

Therefore, several layers of protein-rich corneocytes are surrounded by lipid-rich lamellae membranes and held together with corneodesmosomes in the SC. This highly organized structure is known as epidermal barrier. In a simplified model, the highly organized structure of epidermal barrier is often described as similar to a brick wall, with the corneocytes and lipid lamellae analogous to the bricks and mortar respectively (Figure 1.2). Both the bricks and mortar are produced by granular keratinocytes, which release the lipids mortar into the space between the cells as they are being transformed into the corneocytes bricks. To extend this model, corneodesmosomes link the corneocytes together and act as "iron rods", which pass down through holes in the bricks to give the wall its tensile strength (Getsios et al. 2004; Cork et al. 2006; Naoe et al. 2010). This barrier serves to limit passive water loss from the body, reduce the absorption of chemicals from the environment and prevent the infection of microbial pathogens.

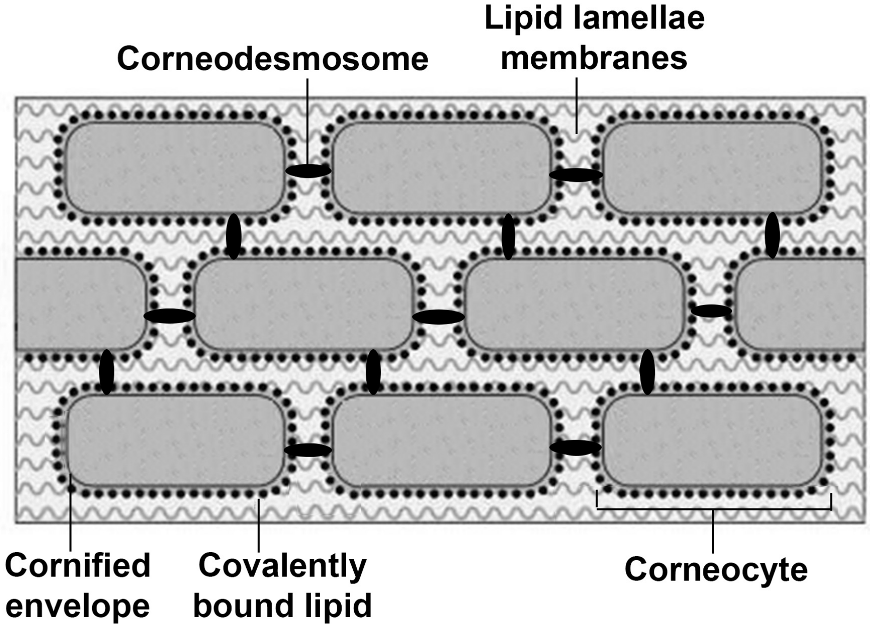

Figure 1.2. The "bricks, mortar and iron rods" model of the epidermal barrier.
The corneocytes represent the bricks and the lamellar lipid represents the mortar. Corneodesmosomes hold the corneocytes together as iron rods. Corneocytes are protected externally by the cornified envelope (CE). One layer of lipids bound to CE covalently, and multiple layers of lipid lamellae membranes fill the spaces between the corneocytes.

The cornified envelope is mainly composed of epidermal structural proteins, such as filaggrin (FLG), loricrin, involucrin, and small proline-rich proteins, which are cross-linked together by the action of transglutaminases. This envelope provides strength to the corneocytes. In addition, FLG and its breakdown products natural moisturizing factors (NMFs) are essential for the retention of water within corneocytes and maintenance of SC hydration (Fuchs and Raghavan 2002; Candi et al. 2005; Segre 2006). The intercellular lipid matrix consists of ceramides (about 50% by mass), cholesterol (about 25% by mass), fatty acids (about 10-20% by mass) (Madison et al. 1987). Thus, non-continuous corneocytes are surrounded by the continuous matrix of specialized lipids, which prevents the development of gaps between the corneocytes and give flexibility to the SC, further enhancing the integrity of the skin barrier. These lipids also help to prevent the escape of internal moisture/water-soluble materials and make the skin resistant to the penetration of allergens and pathogens (Elias 1983; Rawlings 2003; Harding 2004; Proksch et al. 2008). Corneodesmosomes are composed of cell adhesion proteins and linking proteins. The adhesion proteins include transmembrane glycoproteins of the cadherin family, such as desmoglein (DSG) and desmocollin (DSC). Desmoglein 1 (DSG1) and desmocollin 1 (DSC1) constitute extracellular parts of corneodesmosomes. Inside of the corneocytes, DSG1 and DSC1 are connected with intracellular keratin filaments via corneodesmosomal plaque proteins. The cytoplasmic plaques are attached into and become parts of cornified envelopes (Green and Simpson 2007; Garrod and Chidgey 2008; Ishida-Yamamoto et al 2011). These corneodesmosomal proteins are degraded during epidermal desquamation to allow the corneocytes to dissociate and shed from the surface of skin. The processes involved in epidermal desquamation has been described as the network of degradatory proteases, which break down the extracellular domains of corneodesmosomal adhesion proteins, consequently resulting in the dissociation of corneocytes and triggering desquamation (Caubet et al. 2004; Descargues et al. 2006).

1.1.3 The function of epidermal barrier

Epidermal barrier prevents internal water loss and protects our body against the exogenous irritants from the surrounding environment. These defensive functions depend on the integrity of the barrier. Thus, the protective roles of epidermal barrier rely on several components, which determine the formation and maintenance of the SC. For instance, cornified envelope and corneodesmosomes provide the scaffold for structural integrity and mechanical resistance of the epidermal barrier. Moreover, intercellular lipid lamellae are essential for the prevention of transepidermal water loss (TEWL). Furthermore, FLG and NMFs are key molecules maintaining the hydration of the epidermis. FLG is extensively degraded into small peptides and then into free amino acids. The free amino acids are then catabolized into the constituents of NMFs, such as lactic acid, sodium pyrrolidone carboxylic acid, urocanic acid and urea. The NMFs are essential for the retention of water within corneocytes, and results in their optimal hydration and swelling. Sodium pyrrolidone carboxylic acid and lactic acid, in particular, are intensely hydroscopic. They both can absorb water and act as very efficient humectants, which prevents the development of gaps between the corneocytes, enhancing the integrity of the epidermal barrier and making it resistant to the penetration of irritants and allergens (Harding et al. 2000).

In addition, proteases and protease inhibitors can interact with each other to control the rate of skin desquamation. Both exogenous and endogenous proteases are implicated in cleavage of the corneodesmosomal junctions. Among the endogenous proteases, a cocktail of serine and aspartic proteases are secreted into the extracellular space at the SG/SC interface by lamellar granules and participate in the degradation of corneodesmosomes (Ekholm and Egelrud 1998; Ekholm et al. 2000; Bernard et al.

2005). The human kallikreins (KLKs) are main epidermal proteases involved in skin desquamation (Ekholm and Egelrud 1998; Ekholm et al. 2000; Hansson et al. 2002). Other enzyme capable of degrading corneodesmosomal adhesion proteins include aspartate protease, cathepsin D (Horikoshi et al. 1999; Igarashi et al. 2004; Bernard et al. 2005). Research by other groups also revealed the regulatory role of matrix metalloproteases (MMPs) in desquamation (Ohler et al. 2010). The activity of these proteases and the speed of desquamation-related proteolysis are strictly controlled by a complementary cocktail of protease inhibitors. For instance, the lympho-epithelial Kazal-type-related inhibitor (LEKTI) is a particularly important regulator of epidermal desquamation and possesses potent inhibition towards KLKs (Deraison et al. 2007). In addition, human epidermis also expresses the cystatin protease inhibitors, A and M/E, which are specific for cysteine proteases (Zeeuwen et al. 2001).

These factors work together to maintain the integrity of epidermal barrier. Therefore, the barrier formation and function are delicately regulated in healthy skin. However, dysregulation in any of these fundamental components can result in skin barrier dysfunction, consequently leading to a wide range of skin disorders including AD.

1.2 Atopic dermatitis

AD is a common, chronic inflammatory skin disease. The clinical features of AD include xerosis (dry skin), pruritus (itchy skin) and relapsing erythematous lesions (redness of the skin) (Bieber 2008). For a long period, immune dysregulation was considered to be the primary cause of AD, and epidermal barrier impairment was the consequence of inflammatory response. However, immunological dysfunction is not found in all AD patients, suggesting there are some non-immune causative factors that can trigger this disease (Flohr et al. 2004; Bieber, 2008). Some researchers proposed that skin barrier defect could be the initial event but not the secondary phenomenon in AD, which was also supported by further studies (Elias et al. 1999; Taïeb 1999; Elias and Feingold 2001; Flohr et al. 2004; Cork et al. 2006; Callard and Harper 2007; Bieber 2008). For example, variants within genes encoding epidermal barrier structural protein FLG and epidermal protease inhibitor LEKTI can directly result in skin barrier dysfunction in AD, consequently promoting the development of this skin disorder (Chavanas et al. 2000; Kato et al 2003; Nishio et al. 2003; Irvine and McLean 2006; Barker et al. 2007; Brown et al. 2008). In addition, environmental factors such as overuse of soap or detergents can lead to the epidermal barrier defect and trigger the flare-ups in AD (Ananthapadmanabhan et al. 2004; Flohr et al. 2004; Cork et al. 2009). Therefore, AD is increasingly ascribed to primary barrier dysfunction.

1.2.1 Epidemiology

AD affects around 10–20% of children and 1–3% of adults worldwide (Leung et al. 2004). It occurs in any race throughout the world. Genetic variants in diverse populations were found to be associated with the prevalence of AD (Kato et al. 2003; Nishio et al. 2003; Vasilopoulos et al. 2004; Irvine and McLean 2006; Hubiche et al. 2007; Ekelund et al. 2008; Weidinger et al. 2008). Wide variations of AD prevalence have been observed in populations with similar genetic backgrounds within close geographic areas, suggesting the environmental factors may also play a critical role in AD (Leung et al. 2004). The people living in urban area seem to be more susceptible to AD. The prevalence of AD has been rising progressively in industrialized countries since the 1940s and has increased two- to three-fold during the past three decades. In contrast, the prevalence remains much lower in countries with predominantly rural or agricultural areas (Taylor et al., 1984; Williams, 1992; Leung and Bieber 2003; Leung et al. 2004). In addition, another study revealed that the disease burden in cities is much higher compared with the countryside (Schram et al. 2010). These findings suggest that apart from the genetic causes, environmental factor also play an important role in the expression of this disease.

The manifestations of AD usually start and occur during childhood but can still persist or start in adulthood (Spergel 2010). It begins in the first year of life in 60% of cases, and by the age of five years in nearly 85% of cases. AD will clear in almost 40% of patients by adulthood (Williams and Strachan 1998; Williams 2005; Burr et al. 2013). The prevalence data of AD also show a slight female to male preponderance (Williams et al. 1999; Daniels and Harper, 2002; Kang et al. 2003). Therefore, the various observations of the prevalence of AD reveal the complex etiology of this skin disorder, which is possibly triggered by the interaction of multiple causative factors.

1.2.2 Etiology

AD is a multifactorial disease caused by numerous genetic and environmental factors. These factors interact with each other and contribute to the epidermal barrier dysfunction and immune dysregulation in AD. Therefore, various causes work together to determine the severity of AD or the likelihood of developing this skin disorder.

1.2.2.1 Genetic factors associated with AD

AD exhibited genetic linkage to chromosome 1q21. This region consists of the epidermal differentiation complex (EDC), which contains genes regulating epidermal barrier integrity and function. Many research groups have revealed that changes in genes encoding epidermal barrier structural proteins in this region are related to AD (Walley et al. 2001; Vasilopoulos et al. 2004; Palmer et al. 2006; Morar et al. 2007). Among these genes, loss-of-function mutation in the FLG gene was considered to be the most significant genetic factor related with AD (Irvine and McLean 2006; Marenholz et al. 2006; Palmer et al. 2006; Weidinger et al. 2006; Barker et al. 2007; Morar et al. 2007; Brown et al. 2008; Ekelund et al. 2008). The most common variants are R501X and 2282del4, which are carried by about 10% of AD patients of European origin (Barker et al. 2007; Sandilands et al. 2007). Apart from variants in FLG gene, importance of several genes encoding adhesion proteins has been implicated in the maintenance of a functional epidermal barrier. Deficiencies of these proteins may also be associated with the development of AD. Mice lacking DSC1 exhibit a fragile epidermis (Chidgey et al. 2001). There are less desmosomes in desmoplakin knockout mice, which could lead to a significant deficiency of the barrier integrity (Gallicano et al. 1998; Vasioukhin et al. 2001). Furthermore, down-regulated expression of tight-junction proteins Claudin-1, Claudin-23 and a corneodesmosomal component DSG-1 in AD has also been recently reported (De Benedetto et al. 2008).

Variations of genes encoding epidermal proteases have also been found in AD. The gene that has been intensely investigated is human tissue kallikrein 7 (KLK7). It has been reported that gene polymorphism of KLK7 could contribute to the development of AD. In a case–control study on 103 AD patients and 261 matched healthy controls, the results imply a possible association between an AACC insertion in the 3'UTR on the KLK7 gene and AD. Thus, the 4-bp insertion could potentially increase the half-life of KLK7 mRNA, leading to an increased level of this enzyme in the skin of individuals with AD (Vasilopoulos et al. 2004). In addition, transgenic mice overexpressing human KLK7 exhibit enhanced chymotrypsin-like proteolytic activity and develop pathologic changes in the skin similar to those observed in chronic AD, such as increased epidermal thickness, dermal inflammation and severe pruritus (Hansson et al. 2002). Since the initial publication of the AACC insertion of KLK7 gene in AD patients, another two studies have been conducted in European populations. However, the results from these studies indicate that the 4-bp insertion mutation of KLK7 may not be associated with AD (Hubiche et al. 2007; Weidinger et al. 2008).

Variants in genes encoding protease inhibitors are associated with AD as well. Recently, strong association between serine protease inhibitor Kazal-type 5 (SPINK5) gene polymorphisms and AD patients has been revealed by several studies (Chavanas et al. 2000; Kato et al 2003; Nishio et al. 2003). SPINK5 gene encodes LEKTI. LEKTI is a multi-domain serine protease inhibitor, which can target multiple serine proteases in the epidermis. LEKTI is able to inhibit the activity of many epidermal proteases such as trypsin, elastase and shows exceptional inhibitory activity against KLKs (Mitsudo et al. 2003; Paliouras and Diamandis 2006; Fortugno et al. 2011). Loss-of function mutations of SPINK5 and deficiency of LEKTI are found in patients with Netherton Syndrome (NS), a rare but severe autosomal recessive skin disorder. AD shares several clinical features with NS such as atopic manifestations and eczematous lesions. In order to investigate the link between AD and NS, several studies have been carried out to investigate the SPINK5 gene variants and LEKTI expression in both AD and NS patients. One of the most common polymorphisms is G1258A in SPINK5 gene, which can lead to Glu420Lys substitution in LEKTI. Impaired expression of LEKTI protein was found in keratinocytes from AD patients carrying this polymorphism (Walley et al.

2001; Kato et al. 2003; Nishio et al. 2003; Roedl et al. 2009). In addition, another study revealed a frequent and non-conservative LEKTI variant, E420K, impacts on LEKTI function by increasing the likelihood of LEKTI precursor cleavage within the linker region D6-D7. This prevents the formation of the LEKTI fragment D6-D9, which is known to display the strongest inhibitory activity against kallikrein 5 (KLK5) (Fortugno et al. 2012). Therefore, these findings shed light on the importance of LEKTI deficiency and insufficient inhibition of epidermal proteases in the pathogenesis of AD. AD and NS both exhibit epidermal barrier defect, and they share some clinical features including atopy and skin inflammation. Polymorphisms of SPINK5 associated with AD could result in up-regulated activity of epidermal proteases and further result in skin barrier dysfunction, subsequently producing a similar but milder phenotype compared to NS.

Apart from gene variants in SPINK5 gene, a mutation in the cystatin A (CSTA) gene has been identified to correlate with AD. CSTA gene encodes the cysteine protease inhibitor cystatin A, which is able to inhibit the endogenous cathepsins B, -H and –L and the exogenous proteases from house dust mites, such as dermatophagoides pteronyssinus (Der p) 1 and dermatophagoides farinae (Der f) 1. The CSTA gene is located in chromosome 3q21, which has been confirmed as one of the major susceptibility loci for AD (Lee et al. 2000). Decreased expression of cystatin A has been found in the skin of patients with AD (Seguchi et al. 1996). The variant results in decreased mRNA stability and, therefore, decreased levels of the cystatin A within the skin (Vasilopoulos et al. 2007). As a consequence, inhibition of both endogenous and exogenous cysteine proteases is reduced, resulting in the over-degradation of the corneodesmosomes and defect of the epidermal barrier, which subsequently favours the penetration of exogenous pathogens and allergens (Vasilopoulos et al. 2007). Furthermore, transgenic mice carrying a null mutation in the gene encoding cystatin M/E also exhibited severe barrier defect characterized as disturbed cornification and desquamation, and die shortly after birth (Zeeuwen et al. 2001).

In addition, variations of genes encoding cytokines are also correlated with AD. The most investigated immune genes are IL-4, IL-4 receptor alpha (IL4Ra) and IL-13 (Kawashima et al. 1998; He et al. 2003; Kiyohara et al. 2008). IL-4 promotes the development of Th2 cells in allergic inflammation and decreases gene expression in the EDC that contribute to barrier function (Kim et al. 2008; Sehra et al. 2010). IL-13 promotes tissue inflammation and is up-regulated in eczematous skin lesions (Sehra et al. 2010). Multiple SNPs in IL-13 have been significantly associated with eczema in Canadian, Japanese, Dutch, and German populations. IL-4 and IL-13 also share a common receptor subunit, IL4Ra, and SNPs in IL4Ra have also been identified in subjects with AD (Oiso et al. 2000; Callard et al. 2002). The association of TSLP gene polymorphisms with AD has also been reported. TSLP is a pro-Th2 cytokine, which can initiate the differentiation of Th2 cells and promote Th2 cytokine secretion (Liu et al. 2007; Gao et al. 2010; Roan et al. 2012; Bell et al. 2013; Jang et al. 2013; Duchatelet and Hovnanian 2014).

1.2.2.2 Environmental factors relevant to AD

Several environmental effectors on AD have also been reported. For instance, soap and detergents are considered as the most common irritants in contact dermatitis of the hands, and they can also trigger flares of AD (Meding and Swanbeck 1987). In AD patients, skin pH is extremely higher than that of normal donors (Eberlein-Konig et al. 2000). Overuse of soap and detergents is considered to be one of the main causes of increased pH in AD skin (Ananthapadmanabhan et al. 2004). Recent studies indicate that increased skin pH causes hyperactivity of some epidermal proteases which have optimum activity at slightly alkaline pH, resulting in over-degradation of corneodesmosomes and consequently leading to epidermal barrier defect (Flohr et al. 2004; Cork et al. 2009). Furthermore, the acute irritant effects of soap and detergents could be partially explained by their ability to promote the release of pro-inflammatory cytokines from corneocytes (Wood et al. 1996, 1997). In addition to inducing the cytokines release, the use of detergents is also able to cause impaired expression of keratinocyte differentiation markers and epidermal proteases (Torma et al. 2008), consequently exacerbating the skin barrier defect. In addition, the prevalence of AD is much higher in areas where hard water is used to wash the skin compared with areas where the water is soft, which may be attributed to the irritant chemicals in hard water (McNally et al. 1998). The irritation caused by soap, detergents and hard water could contribute to the epidermal barrier dysfunction in AD, subsequently promoting the development of this skin disorder.

As AD is an allergic inflammation, various contact allergens and aeroallergens are considered as potential risk factors in the initiation and aggravation of AD. Proteolytic activity from allergens has been demonstrated to play an important role in the pathogenesis of AD. Exogenous proteases, such as those produced by house dust mites and Staphylococcus aureus (S. aureus), are the common causes of AD (Kato et al. 2005). House dust mites are the source of over 30 different proteins that can trigger immune responses, such as serine and cysteine proteases (Yasueda et al. 1993; Stewart and Thompson 1996). Reports have shown that two proteins called Der p1 and Der p2 derived from house dust mite exhibit proteolytic activity, and these proteins are able to induce immune reactions and further cause skin irritation through their proteolytic activity directly (Deleuran et al. 1998). It has also been revealed that allergens in house dust mite are able to result in the barrier dysfunction and lead to increased penetration of other allergens (Jeong et al. 2008). Furthermore, research by another group revealed that AD patients showed a higher prevalence of mites on their skin compared to healthy individuals, which could be involved in allergic sensitization and disease exacerbation (Teplitsky et al. 2008). S. aureus has been identified as an environmental factor associated with AD since the nineteenth century (Storck et al. 1948). It can produce numerous proteases and may also play a role in the chronicity and severity of AD through the release of superantigenic exotoxins. Apart from their immunological effects, these toxins could also damage the epidermal barrier directly (Leung et al. 1993). In addition, proteases produced by S. aureus are able to degrade AMPs, such as the human cathelicidin LL-37 (Sieprawska-Lupa et al. 2004). Keratinocytes express a number of AMPs when stimulated by microbes or tissue injury. AMPs are responsible for the control of S. aureus and viral replication (Kuo et al. 2013). Deficiencies of AMPs may predispose to microbial colonization and skin inflammation in AD.

AD is an allergic inflammation characterized by Th2 immune response. The level of IgE, which indicates the extent of allergic sensitization, is associated with AD. A high total serum IgE level is a strong risk factor for AD and correlates with the disease severity. IgE-mediated food allergies can trigger the flare-up in AD either through skin contact during food preparation or through food consumption (Laske & Niggemann 2004). Food allergy has been well documented in approximately one-third of children with moderate-to-severe AD. Cow's milk, hen's egg, peanut, wheat, soy, nuts, and fish are responsible for over 90% of food allergy in children with AD. The incidence and type of food allergy can vary with age. In infants, cow's milk, hen's eggs, peanuts, and soy and, in older children, wheat, fish, and tree nuts are the most common food allergens. Adolescents and adults also react to foods, but reactions to 'classical' food allergens such as cow's milk and hen's eggs are not as common as in childhood (Werfel & Breuer 2004). Some children and adults with AD also react to pollen-associated foods (Werfel & Breuer 2004). Birch-associated foods have also been described as potential triggers of AD in children as well as in adults (Vieths et al. 2002). Based on clinical data from the past few decades, it is clear that food allergy can directly provoke flares of AD particularly in sensitized infants, whereas inhaled allergens and pollen-related foods are of greater importance in older children, adolescents and adults (Werfel & Breuer 2004; Bergmann et al. 2013). The diagnosis of food allergy in AD is currently based on the clinical history, skin prick tests, or blood test screening, followed by an elimination diet and/or standardized oral food challenge.

1.2.3 Pathogenesis

Although the pathogenesis of AD is not completely understood, both defective epidermal barrier and abnormal skin immune responsiveness play important roles in the development of this skin disorder. For a long period, AD is thought to be driven by primary immunological dysfunction, and epidermal barrier defect is the secondary consequence of the inflammatory response to irritants and allergens ('inside-to-outside' view of AD pathogenesis) (Leung 2000; Williams 2000; Baker 2006) (Figure 1.3A). At that time, most researchers focused on the immune dysregulation of AD. The infectious agent most often involved in AD is S. aureus, which colonizes in approximately 90% of AD patients (Watson and Kapur 2011). Increased colonization with S. aureus can consequently aggravate barrier function and facilitate microbial invasion in AD through down-regulation of epidermal free fatty acids, which is a key component of the lipid lamellae in epidermal barrier and also exhibits potent antimicrobial activity (Otto 2004; Clarke et al. 2007). In addition, proteases produced by S. aureus are capable of degrading AMPs. The presence of AMPs in the skin provides the first-line of defence against a wide variety of infectious agents. However, the deficiency of these AMPs could facilitate the invasion of exogenous allergens and pathogens, consequently exacerbating microbial colonization and triggering skin inflammation. Apart from the degradation of AMPs (Sieprawska-Lupa et al. 2004), proteases from S. aureus can also promote the premature cleavage of DSG1, subsequently resulting in impaired barrier integrity and contributing to epidermal barrier dysfunction (Hirasawa et al. 2010).

In addition, AD is characterized by predominant Th2 immune response. Previous studies have revealed that exposure to protein antigens results in Th2 immune response and elevated IgE level in AD, which is indicative of immune hyper-reactivity (Williams 2000; Callard and Harper 2007; Bieber, 2008; Newell et al. 2013). Researchers also suggested that the Th2-type inflammation evoked in AD skin could down-regulate the level of lipid production in the epidermis and result in the epidermal barrier dysfunction (Sawada et al. 2012). Furthermore, exogenous application of the Th2 cytokines can lead to decreased amount of corneodesmosomal proteins and abrogate the cohesion of epidermis, subsequently aggravating the epidermal barrier defect (Kobayashi et al. 2004; Hatano et al. 2013). It has also been revealed that Th2-type cytokines are able to inhibit the expression of keratinocyte differentiation-related proteins, most notably FLG, possibly resulting in disorganized keratinocyte growth and disturbed skin barrier formation (Howell et al. 2007). Furthermore, members of two key families of AMPs, LL-37 and beta-defensins are down-regulated in a Th2-dependent manner in AD, which could consequently exacerbates the barrier defect in this skin disorder (Ong et al. 2002; Nomura et al. 2003).

Nevertheless, immune dysregulation is not present in all AD patients, and around 20% of patients are non-atopic and never develop a raised IgE level (Flohr et al. 2004; Bieber, 2008). These findings suggested there are some non-immune causative events in the development of AD, thus other explanations for the pathogenesis of AD were raised. Some researchers thought the epidermal barrier defect was the initial event in AD ('outside-to-inside' view of AD pathogenesis) (Elias et al. 1999; Taïeb 1999; Elias and Feingold 2001; Flohr et al. 2004; Cork et al. 2006; Callard and Harper 2007; Bieber 2008) (Figure 1.3B). Since 1990s, Elias proposed that the epidermal barrier abnormality is not a secondary phenomenon, but rather may be the 'driver' of disease activity in AD (Elias et al. 1999), whereas some scientist were concentrated on immunological dysfunction in AD. A defective epidermal barrier allows the penetration of allergens through the skin, facilitating the interaction of these allergens with the immune cells. In some cases, this may result in the transition from the non-atopic state to the atopic state of the disease with increased IgE level (Novak et al. 2003; Bieber 2008).

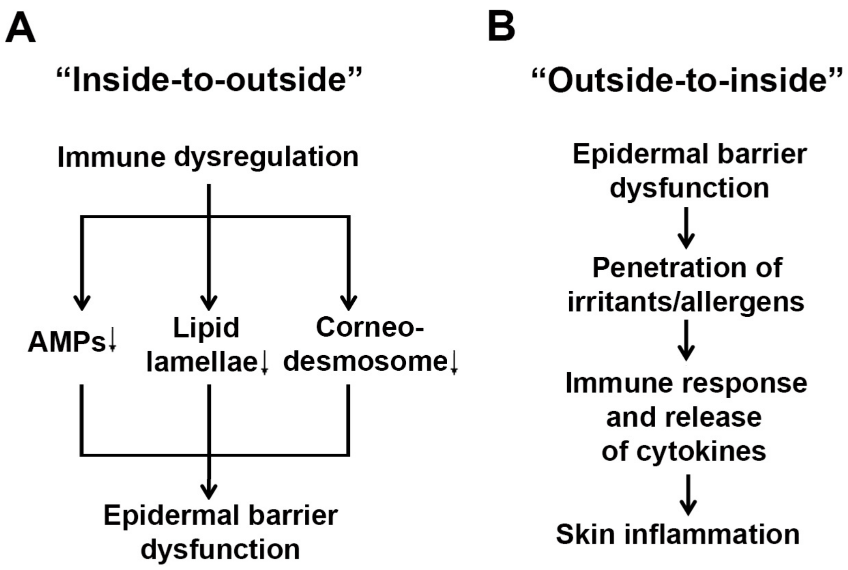

Figure 1.3. Two pathogenetic mechanisms of AD.
AD is considered to be driven by primary immunological dysfunction, and epidermal barrier defect is the secondary consequence of the inflammatory response to infectious agents ('inside-to-outside' view) (A). Recently, some researchers think the epidermal barrier defect is the initial event in AD, which results in the immune dysregulation and skin inflammation ('outside-to-inside' view) (B).

Findings by other research groups also provide evidence to support the 'outside-to-inside' hypothesis. 1) Severity of disease phenotype in AD parallels the extent of the barrier abnormality (Seidenari and Giusti 1995; Angelova-Fischer et al. 2005; Proksch et al. 2006). 2) Clinically nonlesional sites of AD still display barrier abnormalities (Seidenari and Giusti 1995; Bieber 2008). 3) Barrier disruption caused by surfactants (such as sodium lauryl sulfate) or skin stripping leads to the release of cytokines, indicating that barrier damage alone can result in the production of cytokines, skin inflammation and flare of dermatitis (Wood et al. 1996, 1997; Elias et al., 1999). 4) The epidermal barrier disruption contributes to impaired protection against exogenous irritants and allergens, which subsequently results in the development of a Th2 immune response and allergic manifestations (Leung 2013). 5) Restoration of epidermal barrier such as utilization of emollient and specific lipid replacement therapies not only corrects the barrier abnormality, but also reduces the skin inflammation in AD (Chamlin et al. 2002; Grimalt et al. 2006).

Therefore, epidermal barrier dysfunction is likely to be the initial event in AD and may drive the disease activity. Barrier defect can lead to impaired protective function of the skin and allows increased penetration of environmental allergens or pathogens, which may stimulate Th2 immune response and trigger the release of Th2 cytokines, consequently resulting in immune dysregulation and promoting the development of AD.

1.2.4 Epidermal barrier dysfunction in AD

As the pathogenesis of AD is complicated, numerous genetic and environmental causes can contribute to the skin barrier defect in AD. For instance, genetic variations in FLG can result in deficiency or complete absence of FLG expression in AD (Irvine and McLean 2006; Marenholz et al. 2006; Palmer et al. 2006; Weidinger et al. 2006; Barker et al. 2007; Morar et al. 2007; Sandilands et al. 2007; Brown et al. 2008; Ekelund et al. 2008). FLG is a key component of the epidermal barrier. It cross-links with other structural proteins such as loricrin, involucrin and transglutaminases to form the cornified envelope, which contributes to the structural integrity of the epidermal barrier. Furthermore, FLG can be digested by proteolytic enzymes to produce the amino acid components of NMFs. These moisturizers are essential for the retention of water within corneocytes, further maintaining the hydration of SC. Decreased hydration of the epidermis is the first and most obvious cause of barrier dysfunction in AD (Kezic et al. 2008). Mutations in FLG gene can result in the deficiency of FLG protein and its breakdown products NMFs, which consequently contribute to increased TEWL and dehydration of the skin in AD. In addition, as NMFs levels fall, the pH within the epidermis will rise, leading to elevated activity of epidermal proteases especially KLKs, most of which exhibit alkaline pH optima (Brattsand et al. 2005). Although FLG deficiency was considered as a major predisposing factor and closely related to the defective barrier in AD, FLG null mutations may not be sufficient to cause the classic cutaneous abnormalities of AD. Around 50% of individuals with moderate-to-severe AD do not have FLG deficiency (Palmer et al. 2006; Sundberg et al. 2009). Furthermore, Th2-dominant immune responses in AD are not dependant on FLG status (Newell et al. 2013). These findings indicated that apart from FLG, there are other key causative factors that can trigger the skin barrier defect in AD.

Variations within SPINK5 gene could result in deficiency of LEKTI in AD, consequently leading to up-regulated activity of epidermal proteases. For example, G1258A polymorphism has been identified to result in aberrant expressions of barrier-related proteins such as KLK5 and DSG1 (Di et al. 2009). E420K variant in SPINK5 gene results in hyperactivity of epidermal serine proteases. Epidermis with this variant also exhibits increased expression of the proallergic cytokines, reduced level of DSG1 and correlates with accelerated FLG proteolysis, consequently contributing to defective skin barrier permeability (Fortugno et al. 2012). Moreover, SPINK5 knockdown in both organotypic human skin culture and transgenic mice models revealed that LEKTI deficiency results in hyperactivity of epidermal proteases KLK5 and KLK7, which induced premature degradation of corneodesmosomal proteins DSG1, DSC1 and corneodesmosin, further resulting in impaired skin barrier integrity (Yang et al. 2004; Descargues et al. 2005; Wang et al. 2014).

Furthermore, overuse of soap/detergents may lead to increased skin pH and hyperactivity of epidermal proteases, consequently resulting in over-degradation of corneodesmosomes and epidermal barrier defect in AD (Ananthapadmanabhan et al. 2004; Flohr et al. 2004; Cork et al. 2009). Exogenous proteases in the allergens can disrupt the structure and function of epidermal barrier, thereby facilitating further penetration of allergens through the impaired skin barrier and consequently inducing Th2 response (Roelandt et al. 2008). Increased colonization with S. aureus in AD can disrupt the production of epidermal free fatty acids, which is a key component of the lipid lamellae in epidermal barrier and also exhibits antimicrobial activity. As a result, this can lead to epidermal barrier dysfunction and facilitate microbial invasion in AD (Otto 2004; Clarke et al. 2007). Recently, S. aureus has also been reported to produce extracellular V8 protease, which exhibits a similar specificity of glutamate-specific cleavage and a similar sequence to exfoliative toxins and cause epidermal permeability barrier dysfunction in the skin of nude mice by directly degrading DSG1 (Hirasawa et al. 2010).

Many causative factors can contribute to the impairments of skin barrier in AD, such as deficiency of FLG and NMFs, disturbed production of lipid lamellae and up-regulated activities of proteases (Di Nardo et al. 1998; Hara et al. 2000; Kezic et al. 2008; Voegeli et al. 2009). These defects can result in increased risks of skin dehydration and uncontrolled proteolysis of corneodesmosomes, consequently leading to disturbed barrier integrity and higher susceptibility to skin infection. Notably, one of the phenomenons observed in these barrier impairments is up-regulation of epidermal proteases (Roelandt et al. 2008; Di et al. 2009; Cork et al. 2009; Fortugno et al. 2012). Furthermore, enhanced proteolytic activity in the epidermis shows potential to up-regulated proinflammatory cytokines, trigger over-degradation of corneodesmosomal proteins and disturb the processing of FLG and lipid lamellae, consequently contributing to impaired epidermal barrier (Yang et al. 2004; Descargues et al. 2005; Stefansson et al. 2008; Bonnart et al. 2010; Hirasawa et al. 2010; Fortugno et al. 2012; Wang et al. 2014). Therefore, we speculated that up-regulation of epidermal proteases is possibly involved in the development of AD though propagating the vicious cycle of protease-mediated skin barrier defect.

1.3 Epidermal proteases

The balance between epidermal proteases and protease inhibitors is delicately balanced during skin desquamation. However, various genetic and environmental factors involved in AD pathogenesis can result in up-regulated proteolytic activity in this skin disorder. The question of whether unopposed proteolytic activity is a potential accelerator of the skin barrier defect in AD has been raised. Recently, accumulating evidences revealed that up-regulation of epidermal proteases could aggravate skin barrier dysfunction and subsequently trigger the development of AD (Cork et al. 2009).

1.3.1 Proteases and protease inhibitors in the skin

Proteases are proteolytic enzymes that hydrolyse peptide bonds, resulting in irreversible activation or inactivation of the target protein. Until now, members of the four families of proteases have been identified in the epidermis: serine proteases, such as KLKs, matriptase, subtilisins; cysteine proteases, including cathepsin C, L, V and calpains; aspartate proteases, such as cathepsin D and cysteine-aspartic acid proteases; and metalloproteases, including MMP-1, MMP-9, ADAM10, ADAM17 and meprin (Ohler et al. 2010).

Epidermal proteases are involved in the skin desquamation by disassembling corneodesmosomal adhesion molecules. The functional role of epidermal proteases in skin desquamation was first investigated by Bissett et al. They reported that topical application of trypsin or the protease inhibitor could significantly accelerate or reduce the rate of desquamation respectively (Bissett et al. 1987). Subsequently, other researchers also confirmed the contribution of epidermal chymotrypsin-like proteases to skin desquamation (Egelrud and Lundström, 1991; Lundström and Egelrud, 1988, 1990), which was followed by the involvement of trypsin-like proteases in epidermal desquamation (Suzuki et al. 1993, 1996). These endogenous proteases are important regulators of skin homeostasis. In particular, KLKs are abundantly detected in the epidermis, and they account for most of the proteolytic functions in skin desquamation. For instance, kallikrein 5 (KLK5, or stratum corneum tryptic enzyme, SCTE) and kallikrein 7 (KLK7, or stratum corneum chymotryptic enzyme, SCCE) are highly expressed in granular keratinocytes and secreted into extracellular space at SG/SC interface (Caubet et al. 2004). Other studies also indicated that kallikrein 14 (KLK14) was intensively detected in the SG and SC of the epidermis (Brattsand et al. 2005; Borgono et al. 2007).

The activity of the mentioned proteases, and thereby the rate of desquamation, is strictly regulated by a cocktail of protease inhibitors, such as metal ions, lipids, peptides and large protein complexes. They inhibit the activity of the proteases by forming the complexes with them and show inhibitory activity against various epidermal proteases especially KLKs (Goettig et al. 2010). For instance, KLK7 activity is inhibited by the serine leukoprotease inhibitor (Franzke et al. 1996). KLK7 is also inhibited by elafin, otherwise known as skin-derived antileukoprotease, which has been shown to covalently bind to corneocytes (Molhuizen et al. 1993). In addition, α1-antitrypsin and α2-macroglobulin have been reported to be the inhibitors against KLK5 (Yousef et al. 2003a). Human epidermis also expresses the cystatin protease inhibitors, A and M/E, which are specific for cysteine proteases (Zeeuwen et al. 2001). Cystatin A is also secreted in sweat and forms a layer over the surface of the skin that protects the skin from exogenous proteases, such as those produced by house dust mites and S. aureus (Kato et al. 2005). Among these inhibitors, LEKTI is a secreted serine protease inhibitor (serpin) with putative inhibitory activity towards epidermal proteases especially KLKs (Mitsudo et al. 2003; Fortugno et al. 2011). Furthermore, recent studies also showed that LEKTI-2 encoded by SPINK9 and LEKTI-3 encoded by SPINK6 contributed to the control of epidermal KLKs and the regulation of the desquamation process. They are homologues to LEKTI, but contain one Kazal-type domain only (Brattsand et al. 2009; Meyer-Hoffert et al. 2009, 2010).

As each inhibitor acts as a competitive substrate and binds to multiple protease targets, the binding between epidermal proteases and their inhibitors is delicately balanced (Longstaff et al. 1991; Khan et al. 2011). Thus, the activities of epidermal proteases are strictly regulated. Fine-tuned balance between these two groups of enzymes contributes to the maintenance of normal epidermal barrier integrity and function. However, the imbalance between epidermal proteases and their inhibitors can contribute to skin barrier dysfunction.

1.3.2 Up-regulated activity of epidermal proteases in AD

Previous findings suggest that numerous causative factors of AD can lead to barrier defect and consequently result in imbalance between epidermal proteases and protease inhibitors. This subsequently aggravates the barrier dysfunction and increasing the risk of developing AD. Recently, elevated expression and uncontrolled activities of epidermal serine proteases have been found in AD lesions (Voegeli et al. 2009, 2011), suggesting unregulated proteolytic activity may play an important role in the pathogenesis of AD.

As mentioned in Section 1.2.2, both genetic and environmental factors can induce epidermal barrier dysfunction and up-regulated proteolytic activity in AD. For instance, variations in protease genes such as KLK7 and protease inhibitor genes such as SPINK5 may directly result in epidermal barrier defect and up-regulated proteolytic activity in AD (Figure 1.4). In addition, variants within FLG gene can cause decreased levels of NMFs in AD patients, consequently leading to increased pH and elevated activity of epidermal proteases especially KLKs (Brattsand et al. 2005). Moreover, environmental causes of AD such as overuse of soap or detergents and exogenous proteases from house dust mites and S. aureus can also result in skin barrier defect and contribute to increased activity of epidermal proteases. As a result, enhanced epidermal proteolytic activity could lead to over-degradation of corneodesmosomal adhesion molecules such as DSG1, consequently resulting in defective intercellular cohesion in the SC and deficient epidermal barrier integrity in AD skin (Descargues et al. 2005; Di et al. 2009). In addition, increased activity of epidermal proteases is able to induce decreased lipid lamellae synthesis, further exacerbating the barrier dysfunction in AD (Hachem et al. 2006a). These skin barrier defects can lead to increased TEWL and favour the penetration of exogenous pathogens/allergens into the skin.

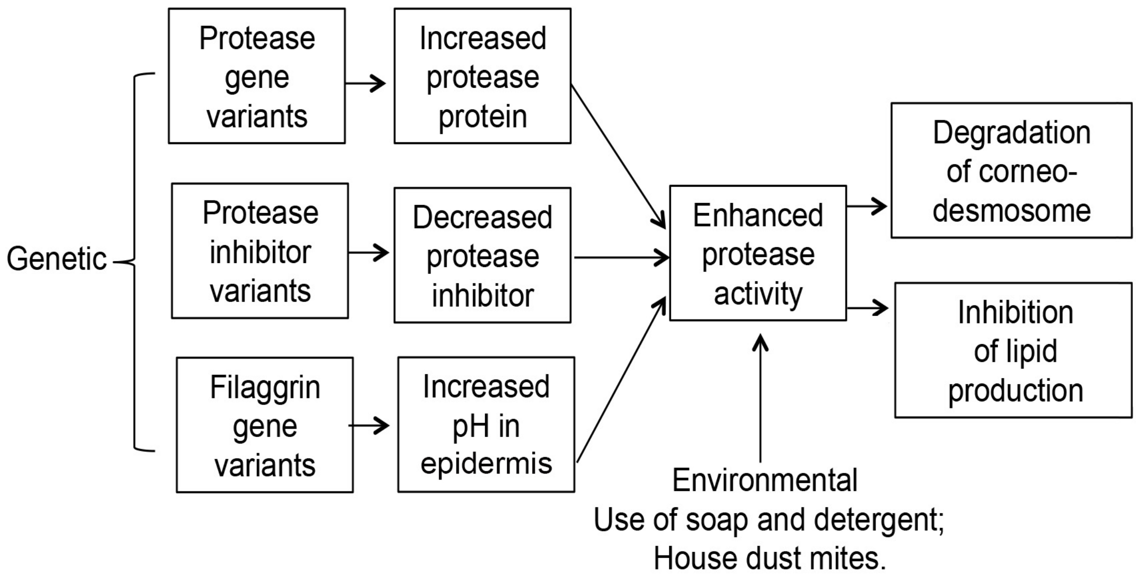

Figure 1.4. The importance of up-regulated protease activity in epidermal barrier defect of AD. *Changes in various genetic and environmental factors contribute to the enhanced epidermal protease activity and exacerbate skin barrier dysfunction in AD (Adapted from Cork et al. 2009).*

Therefore, various causes of AD can contribute to enhanced proteolytic activity, consequently exacerbating the barrier defect and promoting the development of AD. The link between enhanced activity of epidermal proteases and the severity of skin barrier defect has also been revealed in NS, a much more severe condition owing to LEKTI deficiency and uncontrolled proteolytic activity of serine proteases. In "moderate" and "severe" NS, where the activity of serine protease is dramatically increased and extended into the deeper epidermis, corneodesmosomal proteins such as DSG1 are progressively degraded. In contrast, in "mild" NS, lower activity of serine proteases is found within the epidermis, and immunolabeling of DSG1 persists (Hachem et al. 2006b). Interestingly, the G1258A variant within SPINK5 gene, one of the most common polymorphisms in AD and NS, is proved to impact on the function of LEKTI through cleavage on D6-D7 domain. This prevents the formation of the LEKTI D6-D9 fragment that displays strong inhibition against KLK5-mediated degradation of DSG1, subsequently resulting in reduced level of DSG1 (Fortugno et al. 2012). In addition, unregulated activity of serine proteases has been revealed to trigger the overexpression of proinflammatory cytokines through protease activated receptor 2 (PAR2), consequently resulting in the inflammatory response in NS (Briot et al. 2009; Hovnanian 2013). Furthermore, the G1258A variant of SPINK5 gene in AD and NS also leads to the uncontrolled activity of serine protease KLK5 and consequently induces the up-regulation of proinflammatory cytokines (Fortugno et al. 2012). These findings implicate that up-regulated activity of epidermal proteases especially serine proteases potentially aggravates epidermal barrier defect and triggers skin inflammation in AD, subsequently promoting the development of this skin disorder.

1.3.3 Serine proteases kallikreins

Among these above-mentioned epidermal proteases, kallikrein-related peptidases are major and the most intensely investigated proteases in the skin. They are the largest family of trypsin-or chymotrypsin-like secreted serine proteases expressed in the epidermis. KLKs family is composed of 15 members (KLK1–15), and all of them map to the same chromosomal region (19q13.4) in the human genome (Emami and Diamandis 2007). Moreover, KLKs genes share a lot of similar structural features, such as exons and introns organization, conserved intronic intervals, and the lengths of exons (Borgono & Diamandis 2004). KLK5 and the majority of KLKs function as trypsin-like serine proteases, which cleave after arginine or lysine residues. In contrast, KLK7 is a chymotrypsin-like serine protease that cleaves after phenylalanine or tyrosine residues. KLK14 exhibits both activities (Eissa and Diamandis 2008).

KLKs are always co-expressed in certain tissues and coordinate to exert the regulatory roles through proteolytic cascades. The term “KLK activome” was introduced to describe this serial activation of the KLKs zymogens by other mature KLKs. Apart from the cleavage of adhesion molecules such as corneodesmosomal proteins in the epidermis, these activation cascades have also been reported to participate in numerous biological functions such as semen liquefaction, neurodegeneration, and tumor-promoting or -inhibiting effects (Borgono & Diamandis 2004; Yoon et al. 2009).

Until now, eight KLKs have already been identified in the skin, including KLK5, 6, 7, 8, 10, 11, 13 and 14 (Lundwall and Brattsand 2008). KLK5, 7, 8 and 14 seem to be most important,as they are the only KLKs present in active forms in the skin (Ekholm et al. 2000; Brattsand et al 2005; Stefansson et al. 2006; Voegeli et al. 2009). In granular keratinocytes, each of these KLKs is synthesized as inactive pre-pro-enzyme in the endoplasmic reticulum with lengths varying between 244 and 293 amino acids. The synthesized pre-pro-enzyme are transported to Golgi apparatus, where the pre-peptide of 16-33 amino acids is removed. Then they are delivered through trans-Golgi network to lamellar bodies (LB) and secreted to the extracellular space at the interface of granular layer and cornified layer as inactive pro-enzymes. Subsequently, pro-KLKs are activated to mature peptidases by specific proteolytic removal of a pro-peptide (i.e. 37 amino acids in pro-KLK5 and 4-9 amino acids in the other pro-KLKs) from the N-terminus. The removal of N-terminal pro-peptide is mediated via autocatalytic activation (KLK5) or by other KLKs or other endopeptidases in a cascade manner. Present understanding of the activation profiles of KLKs is based on *in vitro* proteolytic cleavage of KLK propeptides and activation of recombinant pro-KLKs. The activation cascades involve a complex network of KLKs (Yousef et al. 2003a; Clements et al. 2004; Emami and Diamandis 2007; Debela et al. 2008; Eissa and Diamandis 2008).

KLKs are key regulators involved in skin desquamation. The proteolytic cascade of KLKs has been proposed in the process of epidermal desquamation, in which activated KLKs are considered as activators of precursors of other KLKs, further resulting in rapid amplification of the overall proteolytic activities (Borgono et al. 2007; Lundwall and Brattsand 2008). Activated KLKs control the rate of desquamation through cleavage of corneodesmosomal proteins (Brattsand et al. 2005; Borgono et al. 2007). As recent studies revealed that KLK5 is able to activate itself as well as pro-KLK2, 3, 6, 7, 11, 12, and 14, KLK5 is believed to be the initiator of the KLKs activation cascade (Brattsand et al 2005; Borgono et al 2007; Lundwall et al. 2008; Yoon et al. 2008; Sotiropoulou et al. 2009). KLK5, 7 and 14 are the most abundant KLKs in the epidermis. They are all produced as inactive precursors, and removal of the pro-peptides leads to the formation of the proteolytically active enzymes (Egelrud and Lundström 1991; Hansson et al. 2002). KLK5 is capable of activating KLK7 (Caubet et al. 2004) and KLK14 (Emami and Diamandis, 2008), in addition to self-activation (Egelrud and Lundström, 1991; Egelrud 1993; Ekholm and Egelrud 1998). After being activated, mature enzymes of KLKs are amenable to inactivation by their endogenous inhibitors such as LEKTI.

LEKTI exhibits putative inhibition against KLKs (Mitsudo et al. 2003; Fortugno et al. 2011). LEKTI is composed 1064 amino acids and 15 serine proteinase inhibitory domains (D1-D15). LEKTI is synthesized in granular keratinocytes as two pro-LEKTI precursors that are 145kDa and 125kDa respectively. These precursors are rapidly cleaved intracellularly by furin in the post-endoplasmic reticulum compartment (Bitoun et al. 2003; Tartaglia-Polcini et al. 2006; Deraison et al. 2007; Fortugno et al. 2011). As LEKTI is a multi-domain protease inhibitor, it has been speculated that LEKTI may target multiple serine proteases in the epidermis. In order to determine the molecular mechanism by which LEKTI functions in the skin, many studies have been carried out. The full-length protein of LEKTI shows no inhibitory activity against KLKs, but is capable of inhibiting trypsin (Paliouras and Diamandis 2006). Another research group has also demonstrated the inhibitory activity of full-length recombinant LEKTI (rLEKTI) against several serine proteases *in vitro*, including plasmin, elastase, trypsin and subtilism A (Mitsudo et al. 2003). Full-length LEKTI is rapidly cleaved into active single and multi-domains intracellularly and secreted into the extracellular space at the interface between SG and SC by lamellar bodies (Bitoun et al. 2003; Ishida-Yamamoto et al. 2005; Jayakumar et al. 2005). Single or multi-domain LEKTI fragments exhibit strong inhibitory activity against KLKs. To date, the inhibitory activity of recombinant LEKTI domains against KLKs has been well established. For example, recombinant LEKTI domain D5, D6, D8-D11 and D9-D15 showed specific inhibition of KLK5, KLK7 and KLK14 (Egelrud et al. 2005; Deraison et al. 2007). Studies by other research groups confirmed the inhibitory efficiency of LEKTI D6-D9 and D9-D12 against KLK5 and KLK7 respectively (Schechter et al. 2005). In addition, LEKTI D12-15 exhibited specific inhibition for KLK5 only (Vitzithum et al. 2008).

In healthy skin, the inhibition of KLKs activity by the serine protease inhibitors occurs through a dedicated balanced inhibitory pathway. However, imbalance between KLKs and their inhibitors can result in epidermal barrier defect.

1.3.4 Up-regulation of KLKs in skin barrier dysfunction

In the epidermis, the activities of KLKs are mainly regulated by LEKTI in combination with changes in microenvironmental pH (Deraison et al. 2007). Furthermore, the cleavage of the corneodesmosomal proteins by KLKs can be inhibited by LEKTI (Borgono et al. 2006).

It has been revealed that KLK5, 7, and 14, together with their inhibitor LEKTI, are all produced in the SG of the epidermis, where the pH is almost neutral and LEKTI binds to KLKs to inhibit their proteolytic activities (Brattsand et al 2005). Recent research also showed that LEKTI is located separately from KLK5 and KLK7 in the lamellar bodies of granular keratinocytes, and secreted earlier than KLK5 and KLK7 to prevent the degradation of corneodesmosomal proteins by KLKs (Ishida-Yamamoto et al. 2005). As KLK5 displays enzymatic activity at the neutral pH of the SG, it is assumed that KLK5 can activate itself in the SG, but its activity is quenched through immediate binding with its inhibitor LEKTI. As a result, KLK5 cannot further trigger the auto-activation and the activation of other KLKs. Consequently, the KLKs activation cascade is quenched and KLKs-mediated proteolytic cleavage of corneodesmosomal proteins including corneodesmosin (CDSN), DSG1 and DSC1 is inhibited.

During the transition of keratinocytes from SG to SC, the pH value drops from neutral to acidic due to the release of free fatty acids. It has been demonstrated that the binding of LEKTI and inactivation of KLKs are reversed by a decrease in pH to the range 4.5–5.5 *in vitro* (Deraison et al. 2007), and SC maintains a pH in this range. Therefore, dissociation of the KLK5-LEKTI complexes and release of active KLK5 enzyme occur as they diffuse into the SC. KLK5 also exhibits enzymatic activity at acidic pH (4.5–5.5) in the SC. Apart from auto-activation, KLK5 can activate KLK7/KLK14 in a cascade manner, and active KLK14 augments KLK5 activity in a feedback loop as shown *in vitro* using recombinant enzymes (Deraison et al. 2007) (Figure 1.5A). As a result, active KLK5, 7, and 14 cause the degradation of CDSN, DSG1 and DSC1, subsequently leading to dissociation of corneocytes and triggering epidermal desquamation (Brattsand et al 2005).

In healthy skin, there is a fine-tuned balance between KLKs and their inhibitor LEKTI, thus normal skin desquamation and epidermal barrier function is delicately maintained (Figure 1.5B). However, the imbalance of KLKs and LEKTI induces over-degradation of intercellular adhesion molecules and leads to premature desquamation. As shown by both *in vitro* and *in vivo* studies, deficiency of LEKTI and unregulated proteolytic activity of KLKs can result in uncontrolled cleavage of corneodesmosomal proteins, subsequently exacerbating the epidermal barrier defect (Descargues et al. 2005).

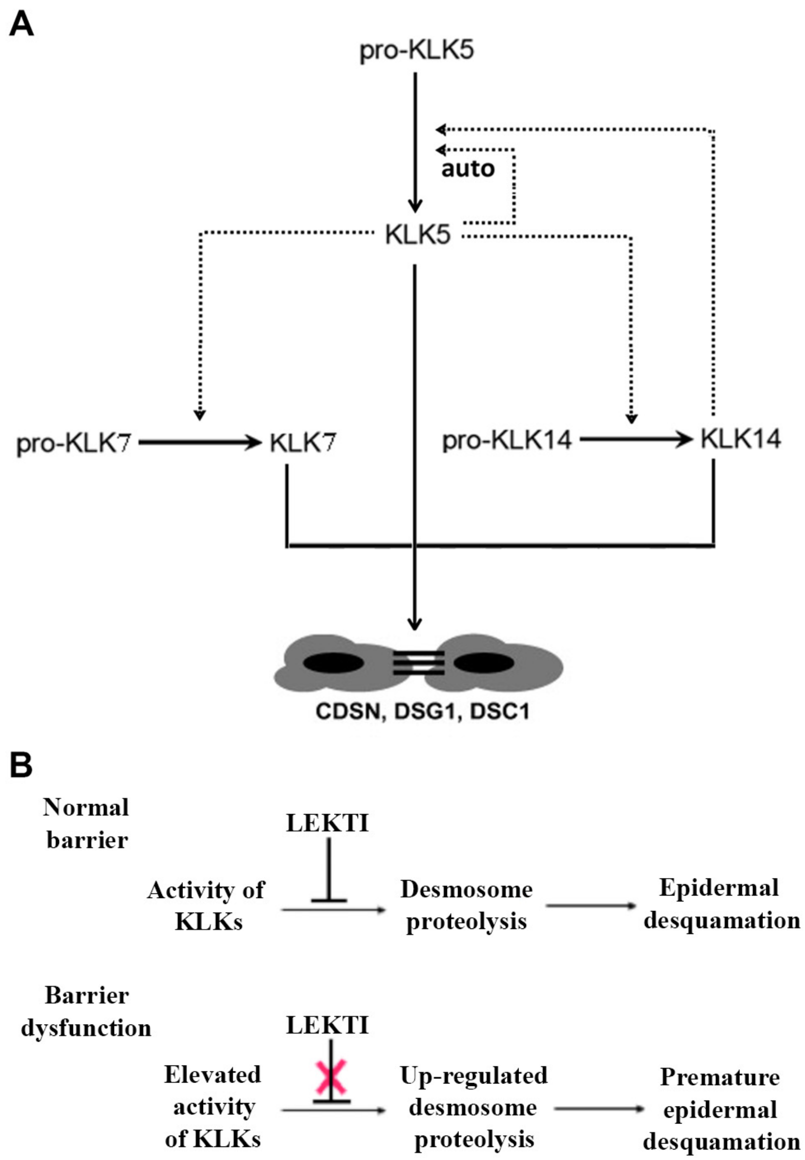

Figure 1.5. Regulatory roles of KLKs and LEKTI in epidermal desquamation.
KLK5 can mediate the activation cascade of KLKs in skin desquamation. Apart from auto-activation, KLK5 can activate pro-KLK7 and pro-KLK14. Active KLK14 is able to activate pro-KLK5. The activated KLKs degrade corneodesmosomal proteins and cause the skin desquamation (A). KLKs and LEKTI are well balanced to maintain epidermal desquamation in normal skin. In comparison, LEKTI deficiency and up-regulated KLKs result in premature desquamation through over-degradation of corneodesmosomes, consequently leading to defective epidermal barrier (B).

KLKs are major proteases expressed in the skin and the key regulators of epidermal desquamation. Up-regulation of KLKs has been found in many skin disorders including AD, NS, and psoriasis, which are all characterised by compromised skin barrier function (Descargues et al. 2005, 2006; Hachem et al. 2006b; Komatsu et al. 2005, 2007a, b; Voegeli et al. 2009). These findings suggest that up-regulation of KLKs may be correlated with epidermal barrier dysfunction. Therefore, we speculated that up-regulated KLKs activity potentially contributed to the skin barrier defect in AD, consequently triggering the development of this skin disease.

Up-regulation of KLKs including KLK5 has been found in the skin samples of AD and NS patients (Table 1.1). Previous studies show increased number of keratinocytes expressing KLK5 mRNA and elevated protein level of KLK5 in the AD skin (Komatsu et al. 2005, 2007b). Another study also revealed elevated proteolytic activity of KLK5 in AD lesions (Voegeli et al. 2009). However, there are some controvertial findings showing that the mass level of KLK5 is not increased in the AD skin (Voegeli et al. 2011). Hyperactivity of KLK5 is also found in NS patients with LEKTI deficiency (Descargues et al. 2005, 2006; Hachem et al. 2006b). In addition, reduced level of LEKTI has also been reported in AD patients (Roedl et al. 2009), indicating that insufficient inhibition of LEKTI could contribute to up-regulated activity of KLK5 in both AD and NS. Furthermore, the G1258A variant in SPINK5 gene, one of the most common polymorphisms in AD and NS, is proved to impact on the function of LEKTI through cleavage on D6-D7 domain. This prevents the formation of the LEKTI D6-D9 fragment, which displays strong inhibition against KLK5, consequently resulting in unopposed activity of KLK5 (Fortugno et al. 2012). These findings suggest the importance of up-regulated KLK5 in the pathogenesis of AD.

Disease	KLKs expression/activity	Up/down-regulation	Reference
AD	mRNA level	KLK1, 4, 5, 6, 9, 10, 11, 13, 14 ↑	Komatsu et al. 2005
AD	Protein level	KLK5, 6, 7, 8, 10, 13, 14 ↑	Komatsu et al. 2007b
AD	Proteolytic activity	KLK5 ↑; KLK7 ↑	Voegeli et al. 2009
AD	Protein level	KLK5 →; KLK7, KLK11 ↑; KLK14 →	Voegeli et al. 2011
NS	Protein level and proteolytic activity	KLK5 ↑; KLK7 ↑	Descargues et al. 2005, 2006
NS	Proteolytic activity	KLK5 ↑; KLK7 ↑	Hachem et al. 2006b

Table 1.1. Dysregulation of KLKs in AD and NS.
Up-regulation of KLKs including KLK5 has been reported in both AD and NS, whereas some study shows unchanged level of KLK5 in the AD skin. ↑: up-regulated. →: unchanged.

1.4 Kallikrein 5

Among the above-mentioned KLKs, KLK5 is one of the major serine proteases abundantly expressed in the skin. It is also known as stratum corneum tryptic enzyme (SCTE) and accounts for most trypsin-like proteolytic activity in the epidermis (Brattsand et al. 2005). Apart from its typical role as a regulator of epidermal desquamation, recent studies have revealed that KLK5 may also involve in other functions of the epidermal barrier, such as modulation of inflammatory response and regulation of skin barrier formation (Briot el al. 2009; Bonnart et al. 2010). Up-regulation of KLK5 has been found in AD (Komatsu et al. 2005, 2007b; Voegeli et al. 2009). As a key epidermal protease and the regulator of skin barrier function, unopposed KLK5 activity may contribute to the epidermal barrier defect in AD, consequently promoting the development of this skin disorder.

1.4.1 Protein structure and enzymatic activity

KLK5 is originally identified as a novel human serine protease in keratinocytes and isolated from human epidermis, which is named as SCTE (Brattsand & Egelrud 1999). According to the new human kallikrein gene nomenclature, the official name is KLK5. Together with the other members of human KLKs family, KLK5 gene has been mapped on chromosome 19q13.4 and constitutes the largest contiguous serine protease cluster in the human genome. The gene is approximately 9.5kb in length, consisting of 6 exons and 5 introns (Brattsand & Egelrud 1999).

KLK5 is a secreted serine protease. In human keratinocytes, KLK5 protein is synthesized as pre-pro-enzyme comprising 293 amino acids, which consists of a predicted signal peptide (29 amino acids), an activation peptide (37 amino acids) and the mature chain (227 amino acids), with 4 potential N-linked glycosylation sites. The serine protease catalytic domain of KLK5 is located in the mature chain. The synthesized pre-pro-enzyme is transported from the endoplasmic reticulum to Golgi apparatus, where the signal peptide is removed. Then they are delivered through trans-Golgi network to lamellar granules, followed by being secreted into the extracellular space in the interface between granular layer and cornified layer as inactive zymogens. Consequently, pro-KLK5 is activated into mature form of the enzyme through proteolytic removal of the activation peptide. The activation of pro-KLK5 has been shown to require the cleavage of an arginine residue (Arg66-Ile67) (Brattsand & Egelrud 1999). KLK5 is capable of auto-activation. Besides of self-activation, pro-

KLK5 could be activated by other serine proteases such as activated KLK14, matriptase and mesotrypsin (Brattsand et al., 2005; Sales et al., 2010; Miyai et al. 2014).

After being activated, mature KLK5 can also activate other pro-KLKs in a cascade manner. Activated KLK5 exhibits potent trypsin-like proteolytic activity by cleaving after arginine or lysine residues in the targets (Michael et al. 2005). In this study using fluorogenic substrates, KLK5 shows preference for AMC conjugated Boc-Phe-Ser-Arg and Boc-Val-Pro-Arg (Michael et al. 2005).

1.4.2 Tissue expression and biological function

KLK5 is widely detected in human tissues and biological fluids. It has been shown to be estrogen/progestin-regulated and highly expressed in endocrine or hormone-responsive tissues, such as prostate, ovary, breast and skin. KLK5 has also been identified in various biological fluids, including seminal plasma, vaginal secretions and breast milk (Brattsand & Egelrud 1999).

Recent studies have revealed that KLK5 is differentially regulated in a variety of hormone-dependent malignancies. KLK5 mRNA and protein levels are significantly increased in patients with ovarian cancer, especially in late stage and higher-grade tumors (Yousef et al. 2003b). Elevated expression of KLK5 is also detected in the serum of patients with breast cancer, and the KLK5 mRNA transcript is found to be an indicator of unfavorable prognosis (Yousef et al. 2003b, 2004). Furthermore, using a human KLK5-specific enzyme-linked immunosorbent assay method, KLK5 has been identified as a potential biomarker for ovarian and breast cancer (Yousef et al. 2003b). In addition, KLK5 mRNA is down-regulated in prostate cancer compared to normal prostatic tissues, and it is a favorable prognostic maker (Korbakis et al. 2009). Aberrant KLK5 expression in tumor tissue may be due to DNA methylation, as reported for KLK6 in breast cancer (Pampalakis and Sotiropoulou 2006). KLK5 splice variants could also result in premature stop of translation or the production of truncated non-functional proteases (Dong et al. 2003; Yousef et al. 2004). However, the roles of dysregulated KLK5 in cancer pathogenesis are still obscure.

KLK5 is involved in semen liquefaction. KLK5 has been shown to auto-activate, and in turn, activates pro-KLK3. Activated KLK3 may contribute to seminal clot liquefaction through hydrolysis of seminal vesicle proteins (Michael et al. 2006). In comparison, the involvement of KLK5 in skin physiology is relatively well established. KLK5 is intensively detected in skin, where it precipitates in epidermal desquamation through cleavage of all three components of corneodesmosomes, including CDSN, DSG1 and DSC1 (Caubet et al. 2004). Although its roles are not completely elucidated, KLK5 may exert physiological functions in various tissues. Therefore, it is considered as one of the most attractive targets to study the pathogenesis of numerous diseases.

1.4.3 KLK5 in the skin

Apart from its well-established function in skin desquamation by degrading corneodesmosomal proteins, KLK5 is also implicated in the proteolytic processing of AMPs such as LL-37 in human skin. KLK5 cleaves the inactive cathelicidin precursor hCAP18 to generate mature peptide LL-37 (Yamasaki et al. 2006). As a result, LL-37 can exert its protective function in defencing the skin against infectious agents from the surrounding environment (Yamasaki et al. 2006; Scott et al. 2007; McGrath & Uitto 2008). Furthermore, accumulating evidences suggest that KLK5 can also promote the inflammatory response in the skin and induce the disrupted formation of epidermal barrier through its downstream molecules (Briot el al. 2009; Bonnart et al. 2010). These findings reveal that KLK5 is a potential regulatory molecule participating in numerous biological functions in the skin.

1.4.3.1 KLK5 induces the activation of PAR2

Protease activated receptors (PARs) are members of the transmembrane G-protein-coupled receptor superfamily. It has been demonstrated that PARs can be activated by proteases through partial proteolytic cleavage on their extracellular domains. PAR2 is a member of PARs family. It is activated by trypsin-like proteases, whereas the other PARs are activated by thrombin (Oikonomopoulou et al. 2006).

Trypsin-like proteases activate PAR2 through proteolytic cleavage at the N terminus R36/S37 activation site of the receptor, leading to the release of a newly formed N terminus (NH_2-SLIGRL), which functions as a tethered ligand to activate the receptor itself (Figure 1.6). Irreversible activation of PAR2 by proteolytic cleavage renders the cleaved receptor unresponsive to further stimulation by activating proteases. Activated PAR2 then trafficks intracellularly, further influencing various biological functions in the cell. Like other G-protein coupled receptors, activated PAR2 is known to have a common signalling pathway, including coupling with G proteins and activation of phospholipase C (PLC). This induces the formation of ionsitol triphosphate (IP_3), consequently triggering the intracellular calcium mobilization (Kawabata 2002; Oikonomopoulou et al. 2006; Stefansson et al. 2008).

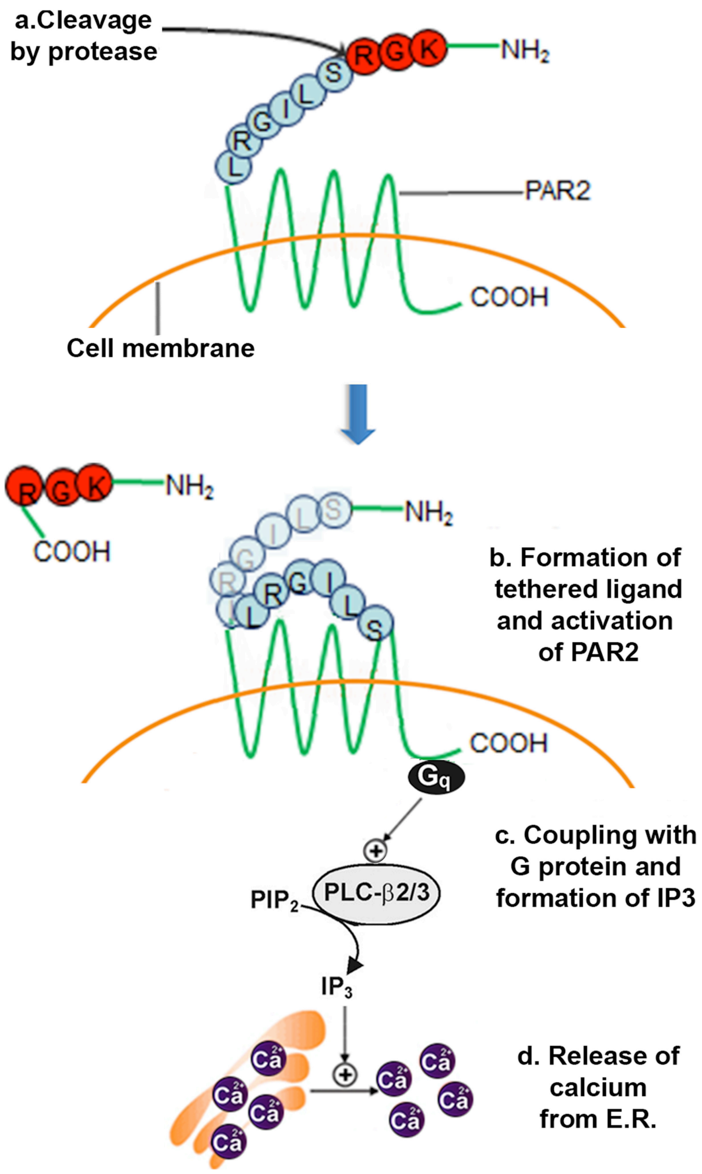

Figure 1.6. Proteolytic activation of PAR2.
Proteases enzymatically cleaves the N-terminal peptide of PAR2 at R36/S37 activation site (a), releasing a newly formed N terminus peptide (NH_2-SLIGRL), which functions as a tethered ligand and triggers the activation of PAR2 (b). Activated PAR2 binds to G-protein and results in the formation of IP_3 (c), further inducing the release of calcium from the endoplasmic reticulum (E.R.) and triggering intracellular calcium mobilization (d)(Reproduced from Kawabata 2002).

PAR2 is present on the membrane of many cells types especially keratinocytes (Santulli et al. 1995; D'Andrea et al. 1998; Steinhoff et al. 1999). The endogenous activators of PAR2 in keratinocytes have been further identified. Potential candidates are (chymo)trypsin-like proteases KLKs, which are also abundantly expressed in keratinocytes. The ability of KLKs was examined with regard to their capacity to activate PAR2 *in vitro*. PAR2 activation was evaluated through measurements of intracellular calcium mobilization. KLK5 and KLK14, which exhibit trypsin-like activity, are both able to trigger PAR2-dependent signaling (Stefansson et al. 2008). These results suggest that KLKs participate in the activation of PAR2 in keratinocytes.

Since previous report implicated that PAR2 is involved in protease-mediated signalling in inflammation and immune response (Steinhoff et al. 2005), PAR2 could play a regulatory role in skin inflammatory reactions after being activated by KLKs. Later on, another study demonstrated that stimulation of human keratinocytes by recombinant KLK5 could induce the activation of PAR2, consequently resulting in up-regulation of proinflammatory cytokines such as thymic stromal lymphopoietin (TSLP) and interleukin-8 (IL-8) (Briot el al. 2009).

TSLP is an epithelial cell-derived cytokine that is involved in the Th2 mediated allergic inflammation. It is a pro-Th2 cytokine that initiates differentiation of Th2 cells and promotes Th2 cytokine responses (Liu et al. 2007; Roan et al. 2012; Bell et al. 2013; Jang et al. 2013). TSLP is considered to be a novel proinflammatory mediator in AD. The importance of TSLP in AD was shown for the first time when increased level of TSLP was detected in lesional skin of AD patients but not in nickel-induced contact allergic dermatitis or in cutaneous lupus erythematosus lesions (Soumelis et al. 2002). Later, another study revealed that overexpression of TSLP in keratinocytes resulted in the development of an AD-like phenotype dominated by Th2 cytokines (Yoo et al. 2005). Recently, the association of TSLP gene variants with AD has also been reported, suggesting TSLP could be a possible causative factor in AD (Gao et al. 2010; Duchatelet and Hovnanian 2014). IL-8 is a chemokine that primarily targets neutrophils, thus it is also known as neutrophil chemotactic factor. Keratinocyte-derived IL-8 has been shown to be a chemoattractant of human neutrophils and T cells, causing the migration of these cells toward the site of inflammation (Barker et al. 1991). Interactions between T cells, neutrophils, and keratinocytes are believed to play a central role in the pathophysiology of inflammatory cutaneous disorders such as AD. These findings provide evidence that keratinocytes could potentially play a key role in mediating the influx of neutrophils and T cells into the epidermis through IL-8 during skin inflammation of AD. In keratinocytes, PAR2 activation leads to increased secretion of IL-8, suggesting the potential role of PAR2 in inflammatory skin disorders (Hou et al. 1998).

Recently, results from another study also indicate that exposure of human skin to irritants triggers up-regulation of KLK5 and activation of PAR2 (Underwood et al.

2013). These findings reveal the potential function of KLK5-PAR2 cascade in the inflammatory response in skin. In addition, PAR2 was also identified as a potential signalling molecule in the epidermal barrier function by regulating the lipid production in the epidermis (Hachem et al. 2006). The lipid bilayers in the SC are key components of the epidermal barrier. However, deficient lipid secretion has been detected in AD patients (Imokawa et al. 1991; Di Nardo et al. 1998; Hara et al. 2000). It has been demonstrated that serine proteases have a central role in the formation and maintenance of the epidermal lipid barrier by degrading the key lipid processing enzymes required for normal permeability barrier homeostasis and PAR2-mediated regulation of LB secretion. Acute barrier disruption leads to an increased pH in the SC, which results in the enhanced activity of serine proteases (Feingold et al. 2007; Demerjian et al 2008). Serine protease hyperactivity in SC can lead to a barrier abnormality via PAR2-induced down-regulation of LB secretion (Hachem et al. 2005, 2006a). As KLK5 is the one of the major serine proteases expressed in the SC, elevated activity of KLK5 could contribute to the impaired lipid processing and lipid barrier abnormalities in AD, consequently exacerbating the epidermal barrier dysfunction during the development of this skin disorder. Furthermore, PAR2 may function as a regulator of keratinocyte proliferation and differentiation (Derian et al. 1997). PAR2 activation is likely to be involved in pruritus of AD (Steinhoff et al. 2003a). PAR2 has been observed to be expressed at high levels in the lesional skin of patients with AD, and PAR2 agonist peptides induce pruritus in AD patients (Steinhoff et al. 2003b). These findings raise the possibility that KLK5 may trigger the inflammatory response and disturb the epidermal barrier formation through activation of PAR2.

1.4.3.2 KLK5 cleaves corneodesmosomal protein DSG1

KLK5 is a key serine protease involved in desquamation with trypsin-like activity (Egelrud and Lundström 1991; Egelrud 1993; Suzuki et al. 1994; Ekholm and Egelrud 1998; Hansson et al. 2002). In healthy skin, KLK5 has been shown to cleave corneodesmosomal adhesion molecule DSG1. Both KLK5 and DSG1 are located in the outermost layers of epidermis, where KLK5 can specifically cleave the adhesive extracellular domain of DSG1 to maintain epidermal barrier integrity in the superficial layer (Caubet et al. 2004; Descargues et al. 2006). However, up-regulated KLK5 activity can cause over-degradation of DSG1, leading to premature desquamation and epidermal barrier dysfunction. It has also been revealed that the activity of KLK5 is increased in NS patients and models with LEKTI deficiency, which leads to reduced level of DSG1, consequently resulting in atopic manifestation (Descargues et al. 2005, 2006; Di et al. 2009; Roedl et al. 2009). Moreover, over-degradation of DSG1 by up-regulated KLK5 can be inhibited by LEKTI (Borgono et al. 2007; Fortugno et al. 2011). Recent study also demonstrated that SPINK5 and KLK5 double knockdown in organotypic human skin culture could restore the level of DSG1 (Wang et al. 2014). These findings indicate that up-regulated activity of KLK5 can result in progressive cleavage of DSG1, consequently leading to premature desquamation and deficient epidermal barrier integrity.

In addition to serving as a rigid anchor between adjacent cells, recent study implicates DSG1 as a key component of a signalling axis governing epidermal morphogenesis. Distinct from other desmosomal cadherins, DSG1 is first expressed at the interface between basal and suprabasal layers, where keratinocytes commit to differentiation (Green and Simpson 2007; Garrod and Chidgey 2008). These findings raise the possibility that DSG1 could also serve as key morphoregulator in the epidermis. It has been demonstrated that DSG1 was required to down-regulate epidermal growth factor receptor (EGFR) signalling and promote epidermal differentiation, indicating DSG1 is also a potential regulator in keratinocyte morphogenesis (Getsios et al. 2009). DSG1 is a target molecule of KLK5. Although KLK5 is abundantly detected in the upper epidermis (Komatsu et al. 2005), there could also be KLK5 expression in the inner epidermal layers, where KLK5 may interact with DSG1 and further involve in the regulation of keratinocyte growth through DSG1. It has been reported that AD exhibits disorganized keratinocyte growth. Increased proliferation accompanied by disturbed differentiation has been found in AD skin (Proksch et al. 2006). These findings raised the possibility that up-regulated KLK5 might contribute to disrupted keratinocyte proliferation and differentiation in AD via DSG1.

1.4.3.3 KLK5 participates in FLG and lipid processing via ELA2

Recently, elastase 2 (ELA2), a novel epidermal protease, has been identified in human and mouse skin and is considered to be a potential trigger in NS pathogenesis through misprocessing of FLG and lipid. In a previous study, it has been confirmed that KLK5 is able to activate ELA2 through proteolytic cleavage of pro-ELA2, and co-incubation of pro-ELA2 with KLK5 results in hyper-activity of ELA2 (Bonnart et al. 2010). Furthermore, hyper-active KLK5 could contribute to the enhanced activity of ELA2 in the LEKTI-deficient epidermis of both NS patients and SPINK5 knockout mice

(Bonnart et al. 2010). This study also suggested that LEKTI was able to inhibit the activity of ELA2 by down-regulating KLK5-mediated activation of pro-ELA2 (Bonnart et al. 2010).

Moreover, ELA2 is found to be co-localized with (pro)-FLG in the epidermis, and it can directly proteolyze both pro-FLG and FLG monomers (Bonnart et al. 2010). FLG itself contributes to the epidermal barrier integrity by aggregating the keratin interfilaments to form the cornified cell envelope, thereby providing the structural support and mechanically resilient skin barrier. In addition, FLG can be degraded into highly hygroscopic amino acids, NMFs, and contribute to water retention within the SC layers, helping to maintain skin hydration (Scott et al. 1982; McGrath & Uitto 2008). Therefore, hyperactive KLK5 results in enhanced activity of ELA2, which could induce misprocessing of (pro)-FLG. As a result, FLG deficiency leads to defects in the formation of cornified envelope, a decreased hydration in the SC and a concomitant elevation of pH. In addition, ELA2 hyperactivity is sufficient to induce the disruption of the lipid lamellae formation (Bonnart et al. 2010). These findings suggested that up-regulated KLK5 activity could contribute to the deficiency of FLG and lipid secretion through activation of ELA2, which leads to increased water loss and lipid lamellae defect. Many of the above-mentioned functional studies of KLK5 were carried out in NS model, where uncontrolled KLK5 activity potentially aggravates epidermal barrier dysfunction and results in AD-like lesions through PAR2, DSG1 and ELA2. Although the etiologies are different, NS and AD are both characterised with defective skin barrier and share several clinical features such as eczematous lesions and atopic manifestations. Therefore, the mechanisms of atopic manifestation in NS caused by up-regulated KLK5 may also be implicated in the more mild and common disease, AD.

1.4.4 Possible roles of up-regulated KLK5 in AD

Up-regulation of KLK5 has been found in AD patients (Komatsu et al. 2005, 2007b; Voegeli et al. 2009). As AD is a chronic skin disorder, many genetic and enviromental factors may lead to persistent epidermal barrier defect in AD, consequently resulting in consistently up-regulation of KLK5. For instance, variants within LEKTI gene could induce lack of inhibition against KLK5 and lead to sustained up-regulation of KLK5 in AD. In addition, variations of FLG gene and overuse of soap both result in elevated pH in the epidermis (Ananthapadmanabhan et al. 2004; Brattsand et al. 2005), which could subsequently induce consistently increased KLK5 activity in the AD skin.

Despite the well-established functions of KLK5 in epidermal barrier through PAR2, DSG1 and ELA2, the association between KLK5 up-regulation and skin barrier defect is still obscure and needs to be fully elucidated. We speculated that up-regulated KLK5 could contribute to the epidermal barrier dysfunction in AD through its downstream molecules (Figure 1.7). Previous studies have reported that single stimulation by KLK5 triggers over-expression of proinflammatory cytokines in keratinocytes through PAR2 (Stefansson et al. 2008; Briot et al. 2009). However, exposure to PAR2 stimuli at a supramaximal concentration or repeated stimulation could cause desensitization and impaired function of PAR2 (Dery, O. et al. 1998; Oikonomopoulou, K. et al. 2006). It remained unclear whether the function of PAR2 can be affected by repeated stimulation of consistently up-regulated KLK5 in AD, and what the potential influences on proinflammatory cytokines are. In addition, up-regulated KLK5 results in over-degradation of DSG1 (Descargues et al. 2005, 2006; Di et al. 2009) and may also contribute to disorganized keratinocyte growth through DSG1. This could lead to impaired barrier integrity and disrupted barrier formation, consequently exacerbating skin barrier defect in AD. Furthermore, up-regulated KLK5 leads to FLG deficiency and lipid defect through activation of ELA2 (Bonnart et al. 2010), which may disturb the barrier integrity and subsequently aggravate the epidermal barrier dysfunction in AD. Therefore, KLK5 could be a potential therapeutic target in AD. More importantly, inhibition of up-regulated KLK5 may improve the skin barrier function, making it a promising therapeutic intervention for AD.

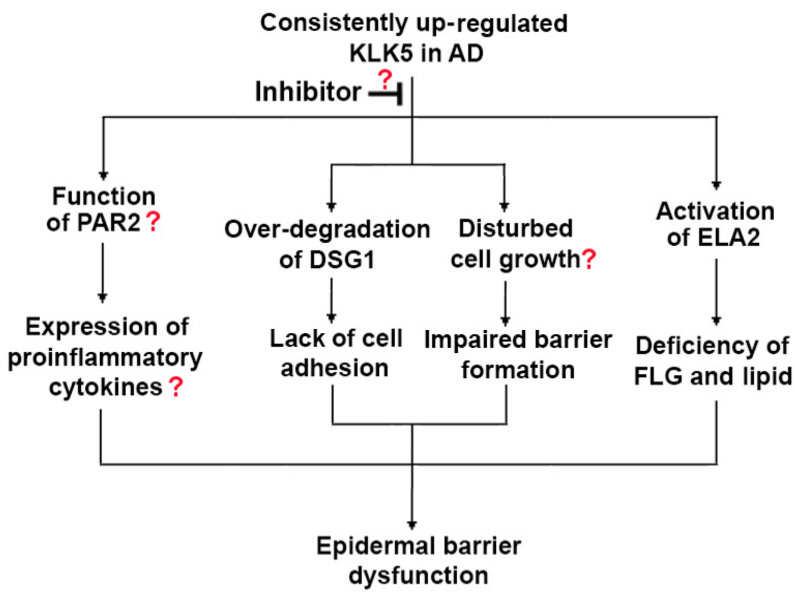

Figure 1.7. Schematic diagram of the possible mechanisms underlying epidermal barrier defect in AD triggered by up-regulated KLK5.

Consistent up-regulation of KLK5 may exacerbate the skin barrier dysfunction in AD through PAR2, DSG1 and ELA2. The epidermal barrier may be repaired through inhibition of unregulated KLK5 using potential inhibitor.

1.5 Treatment for AD

AD can impact the quality of life of affected individuals significantly. Apart from the visible skin conditions, patients with AD suffer from intense itch, which can lead to sleep disturbances. Children with AD have daytime behavioural difficulties associated with insufficient sleep (Chamlin et al. 2005). Recent studies also reveal that quality of life can be affected by AD (Yaghmaie et al. 2013). These findings suggest the necessity to improve the quality of life of AD patients by managing this disease effectively. In addition, the financial impact of AD can also be great. Patients and their families are burdened with time-consuming treatments for the disease, as well as the dietary and household changes. Furthermore, treatments for AD require frequent application of topical medications, which also poses a significant economic burden for the healthcare system (Carroll et al. 2005). The general clinical situation is accentuated by the large population of patients with AD. Therefore, exploration of new therapeutic strategies for AD could be beneficial for patients, families and the society. The aim to get a precise understanding of the role of KLK5 up-regulation in skin barrier impairment is to find more effective therapeutic strategies for AD, such as inhibition of unopposed KLK5 activity using potential inhibitor.

1.5.1 Management and current therapies for AD

Appropriate daily skin care is vital in the management of AD. Avoidance of exposure to environmental irritants and allergens is advised for the patients. All cotton clothing and bedding that is less irritating than synthetic or wool fabrics should be used for affected individuals (Mason 2008). Moisturizing cleansers are recommended while highly fragranced soaps should be avoided as they may irritate the skin (Watson and Kapur 2011). After bathing, moisturizers and emollients should be applied liberally to help prevent moisture loss and drying of the skin (Watson and Kapur 2011). In addition, antihistamines may be helpful especially at night to control the intense itching associated with AD (Herman and Vender 2003).

At present, most of the treatments for AD aim at reducing the clinical symptoms of the disease. Anti-inflammatory therapies such as utilization of steroids and immunomodulators remain the first-line pharmacologic treatments for AD, including topical and systemic application of corticosteroids. Recent studies reveal that topical application of immunomodulators could not only suppress immune function, but also compromise epidermal barrier function (Kao et al. 2003; Choi et al. 2006; Kim et al.

2010). However, the usage of topical immunosuppressants have raised safety concerns, such as increased risk of serious infectious complications and UV-induced skin cancer at sites of prolonged applications (Paller et al. 2001; Soter et al. 2001; Wahn et al. 2002 Hickey et al. 2005; Elias and Wakefield, 2011). Xerosis is another clinical features of AD, and emollient moisturizers can also ease the symptoms of AD through moisturization (Rawling et al. 2004; Korting et al. 2010). Although these therapies can reduce the disease severity in AD, the primary barrier defect that 'drives' the development of AD is not completely corrected (Elias et al. 2008).

Previous studies in animal models and patients of AD indicate that barrier repair interventions can improve epidermal barrier function and further reduce the skin inflammation (Elias 2008; Ong 2009). For instance, lipid replacement therapies have been used to repair the skin barrier in AD. However, the non-physiologic lipids in these moisturizers can impair the epidermal barrier rather than correcting the barrier function (Elias 2006). The defect of epidermal barrier in AD is characterized by deficient lamellar secretion and reduction of all three types of key lipids, cholesterol, free fatty acids, and ceramides, with a significant deficiency of ceramide content. Ceramide serves as the major water-holding molecule in the extracellular space of the SC, and reduced level of ceramide was found in AD patients (Imokawa et al. 1991; Di Nardo et al. 1998; Hara et al. 2000). Thus, correction of the barrier dysfunction in AD requires topical applications not only of sufficient quantities of lipids but also the lipids with a dominant proportion of ceramide (Elias 2006). Recent studies reveal restoration of epidermal barrier in AD by lipid replacement therapy with a ceramide-dominant form (Elias 2008).

1.5.2 Development of AD treatments

Apart from the existing treatments, potential therapeutic approaches for AD such as application of specific siRNA and inhibitors have also been evaluated. Previous report reveals that non-invasive delivery of anti-IL-10 siRNA into the epidermis significantly reduced the mRNA level of IL-10, which is elevated in AD skin lesions (Ohmen et al. 1995; Kigasawa et al. 2010). Another study investigated the transdermal delivery of siRNA against the NFkB subunit RelA, a possible therapeutic target in AD (Uchida et al. 2011). Previous findings also suggest that specific inhibitors of serine proteases chymases and phosphatase calcineurin are potential therapeutic agents for AD (Imada et al. 2002; Terakawa et al. 2008; Carr 2013). In addition, UV phototherapy could be beneficial for the adult patients of AD. However, the usefulness of this therapy is limited due to the potential toxicity of long-term UV therapy, inconvenience and expense (Watson and Kapur 2011).

At present, the therapies of AD continue to be dominated by proof-of-concept studies of primarily anti-inflammatory medications. Although always effective, current treatments have the same problems in AD as they do in other chronic inflammatory dermatoses such as psoriasis: long-term effects associated with toxicity and potential serious side effects. The effective treatment of AD requires multi-pronged approaches, which include the combination of skin barrier repair, control of skin inflammation and elimination of exacerbating factors. Although much can be managed with existing therapy, AD continues to present an unmet need for safe and effective therapy (Arkwright et al. 2013). In order to explore the potential treatment options for patients suffering from this skin disorder, intensive studies are carried out to understand the mechanisms underlying the pathogenesis of AD, which is still not fully elucidated. As AD is increasingly ascribed to a primary epidermal barrier dysfunction, therapeutic strategies of AD should be first directed at improvement of the epidermal barrier. However, specific aspects of AD therapies such as restoration of skin barrier function remain largely untargeted. Therefore, the mechanisms underlying impaired epidermal barrier in AD need to be further investigated.

1.5.3 Inhibition of up-regulated KLK5 as a potential therapy for AD

As a chronic skin disorder, AD is characterized by persistent epidermal barrier defect, which may induce sustained up-regulation of KLK5. Considering the regulatory roles of KLK5 in skin barrier function through PAR2, DSG1 and ELA2 (Section 1.4.3), unopposed KLK5 activity might aggravate the epidermal barrier dysfunction in AD through its downstream molecules, consequently promoting the development of this disease. Therefore, KLK5 could be a promising therapeutic target in the treatments of AD. Recently, several proteases were selected as molecular targets for drug design. The inhibitors of numerous proteases such as MMPs, serine proteases and aspartic proteases have been exploited as therapeutics and eventually entered the pharmaceutical market (Sotiropoulou & Pampalakis 2012). As dysregulation of various proteases were implicated in the pathogenesis of numerous diseases such as HIV and cancer, proteases are attractive targets in the drug development for these diseases (Pandey et al. 2007; Fayard et al. 2009; Llibre 2009). Among these proteases, KLKs represent a major proteolytic system operating in many tissues, but their biological roles are still not well defined. An increasing number of studies implicate that aberrant regulation of KLKs is associated with diverse diseases such as hypertension, skin disorders, inflammation and cancer and suggest their clinical applicability as disease biomarkers (Yousef et al. 2003b; Michael et al. 2006; Pampalakis & Sotiropoulou 2007; Korbakis et al. 2009). In addition, KLKs have emerged as versatile signalling molecules. Therefore, KLKs represent attractive biomarkers for clinical applications and potential therapeutic targets for common human pathologies (Sotiropoulou & Pampalakis 2012). Identification of potent specific and selective inhibitors of KLKs mainly through high-throughput screening platforms and substrate-guided design will aid the development of novel KLKs activity-modulating agents and the discovery of presently unidentified pathways mediated by KLKs in vivo (Swedberg et al. 2009; 2011; De Veer et al. 2013). In conclusion, inhibition of up-regulated KLKs, such as KLK5, for the treatments of diseases including AD represents a largely unexploited area that will be considerably developed in the future.

Sunflower trypsin inhibitor (SFTI-1) is a 14 amino acid cyclic peptide extracted from the sunflower seeds. It exhibits exceptionally potent trypsin-inhibitory activity. SFTI-1 and SFTI-1-derived analogues are known to act upon a wide range of serine proteases including cathepsin G7, matriptase and KLKs (Long et al. 2001; Swedberg et al. 2009; De Veer et al. 2013; Tan et al. 2013). In this thesis, Dr. Macmillan's group at UCL Chemistry Department demonstrated that the circular peptides could be prepared via a native chemical ligation-type process. This strategy was utilised for the first time to produce the analogues of SFTI-1. Ile10 in the scaffold of SFTI-1 was substituted with glycine, which had been demonstrated to give rise to a folded SFTI-1 analogue. The synthesised peptide was designated as SFTI-G and exhibited inhibitory activity towards commercial recombinant KLK5 in spectrofluorimetric assay. Further experiments were conducted in this project to evaluate the influences of SFTI-G on the biological functions of KLK5 in human keratinocytes. Previous findings suggest that up-regulated KLK5 could exacerbate the epidermal barrier dysfunction in AD. An improved understanding of the contribution of KLK5 up-regulation to skin barrier defect and the development of AD could facilitate the drug development for this skin disorder. Therefore, restoration of epidermal barrier function through inhibition of unopposed KLK5 activity is a promising therapeutic intervention for AD.

1.6 Hypothesis and objectives

1.6.1 Hypothesis

In this project, it was hypothesized that up-regulation of KLK5 could exacerbate the epidermal barrier dysfunction in AD through affecting the cytokine production and cell growth in keratinocytes. If this is the case, inhibition of unopposed KLK5 activity might improve the skin barrier function, which is a promising therapeutic intervention for AD.

1.6.2 Objectives

The major objectives of this project are as follow,

1. To compare the epidermal morphology, expression/activity of KLK5 and levels of relevant barrier-related proteins in normal and AD skin.

2. To investigate the contribution of up-regulated KLK5 to skin barrier defect, keratinocytes ectopically overexpressing KLK5 (KLK5-cells) could be generated using lentiviral vector.

3. To further study the influences of overexpressed KLK5 on epidermal barrier function, using *in vitro* skin-equivalent organotypic cultures (OTCs) and *in vivo* skin grafts generated from KLK5-cells.

4. To investigate the effects of consistently up-regulated KLK5 on cytokine production and cell growth in keratinocytes, expression of related genes and proteins in KLK5-cells.

5. To verify whether the impairments caused by up-regulated KLK5 can be reversed through inhibition of unopposed KLK5 activity in KLK5-cells treated with SFTI-G.

Specific studies have been planned for each chapter, and a brief description is added per chapter.

CHAPTER 2: METHODS

2.1 Recruitment of patient samples

Clinical material was obtained with written informed consent from patients attending dermatology clinics at Great Ormond Street Hospital; ethical approval was granted by the local research ethics committee. Skin biopsies were taken from the nonlesional and lesional sites of five AD patients and five age-matched healthy donors. Each individual was given written informed consent before the biopsy was taken. The skin samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, Poole, UK) overnight at 4°C and embedded in paraffin. All paraffin-embedded tissue was cut into skin sections (5µm thickness) and collected onto Superfrost plus slides (Fisher Scientific, Loughborough, UK). For the skin cryosections, the samples were snap frozen in liquid nitrogen in Tissue-Tek O.C.T. (Thermo Fischer Scientific, Loughborough, UK) and cut into sections (5µm thickness).

2.2 Staining of skin biopsies

2.2.1 Histological staining

Hematoxylin and eosin staining (H&E staining) was performed on paraffin-embedded sections using standard histological techniques. The skin sections were deparaffinized in two changes of 100% xylene for 5 minutes each. Then the sections were rehydrated in 100% ethanol twice and 70% ethanol twice for 5 minutes each. The slides were then kept in tap water for 5 minutes. The sections were stained with hematoxylin (GHS1128, Sigma-Aldrich, Poole, UK) for 10 minutes and briefly rinsed in tap water. Excess staining was removed by quickly dipping the slides in acidic ethanol (1% HCl in 70% ethanol) and rinsed again in tap water. Then the sections were stained in eosin (E4009, Sigma-Aldrich, Poole, UK) for 2 minutes. Stained sections were dehydrated in two washes of 70% ethanol, 100% ethanol, 100% xylene for 5 minutes each and mounted with DPX solution (Leica Biosystems, Newcastle, UK).

2.2.2 Immunostaining

Immunostaining for KLK5, PAR2 and FLG was performed on paraffin-embedded sections. The skin sections were dewaxed in two washes of 100% xylene for 5 minutes each. Then the sections were immersed in 100% ethanol twice and 70% ethanol twice for 5 minutes each. Antigen retrieval was achieved by microwave treatment in freshly prepared 10mM sodium citrate (pH 6.0) for 5 minutes 3 times. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in 1 x phosphate buffered saline

(PBS). Tissues were then incubated in fetal calf serum (FCS) for 30 minutes at room temperature. The sections were incubated with primary antibodies in a humid chamber overnight at 4°C. All the primary antibodies were diluted in PBS, and information and the dilutions of the primary antibodies used for immunostaining were listed in Table 2.1 (Section 2.17). Negative controls were included for each sample by omitting the primary antibody.

Next day, primary antibodies were removed by three washes with PBS for 5 minutes each. Tissues were incubated with appropriate biotinylated secondary antibodies (Dako, Cambridgeshire, UK) in blocking buffer (1 x PBS with 0.1% Triton X-100 (Sigma-Aldrich, Poole, UK) and 3% FCS) with the dilution factor of 1:100. Tissues were rinsed in PBS for three times and incubated with VECTASTAIN Elite ABC reagent that contains equal amount of Reagent A and B (Vector Laboratories, Peterborough, U.K.) for 30 minutes, and then developed with Vector DAB Peroxidase Substrate (Vector Laboratories, Peterborough, U.K.). Nuclei were counterstained with hematoxylin. Stained sections were dehydrated and mounted with DPX solution (Leica Biosystems, Newcastle, UK).

Immunostaining of DSG1 was performed on skin cryosections. Prior to fixation, sections were air-dried and washed twice in PBS. Fixation of the tissue samples was performed by immersing the sections in ice-cold acetone (Sigma-Aldrich, Poole, UK) for 10 minutes. After fixation, the sections were washed three times in PBS. Then tissues were incubated in blocking buffer for 30 minutes at room temperature prior to incubation with primary antibodies overnight at 4°C. The following day, after several rinses with PBS, sections were incubated with appropriate Alexa-Fluor 488 or 568 conjugated secondary antibody (1:500, Molecular Probes, Eugene) in dark for 1 hour at room temperature. All negative controls were incubated in the presence of secondary antibody alone. Nuclei were counter-stained with DAPI (Vector Laboratories, Peterborough, U.K.). After rinsing with PBS, the sections were mounted using Mowiol solution containing 10% Mowiol D488 (Calbiochem, Nottingham, U.K.), and the slides were kept at -20°C.

Stained sections were visualized using a Leica DMLB Microscope. Images were captured by a microscope-mounted CCD camera (Cool-SNAP-Pro; Media Cybernetics, USA) controlled by Image-Pro Plus, version 6.0 (Media Cybernetics, USA). All images were taken with the same setting and saved as 24-bits color images or 8-bit gray-scale images.

2.2.3 Quantification of staining intensity

Quantification of protein expression in the epidermis of stained skin sections was performed using computerised software Image-Pro Plus v6.0 (Media Cybernetics Corporation, USA). The images of three non-overlapped but adjacent regions in each skin section were recorded and saved digitally. In order to ensure the relative reliability, the light level on the microscope was set to a fixed value and the camera acquisition settings were kept constant to capture the images.

The intensity threshold of positive staining for each antibody was defined and adjusted using the eyedropper tool provided in the software, and DAB-stained dots with brown color were selected (Figure 2.1). Color signal above the threshold for each antibody was considered to be positive, whereas any signal below the threshold was regarded as negative. The defined color threshold was saved and further applied for the stained skin sections. Before automate counting, the epidermis in each region was highlighted manually as an area of interest (AOI) using the irregular tool (Figure 2.1). The optical counts of positive staining within AOI were measured based on the defined threshold. The staining intensity in each AOI was shown as mean density, which was calculated as integrated optical density (IOD)/area. Average intensities of the positive staining in normal, AD nonlesional and AD lesional skin were analyzed using one-way ANOVA. The differences were considered statistically significant at $p<0.05$.

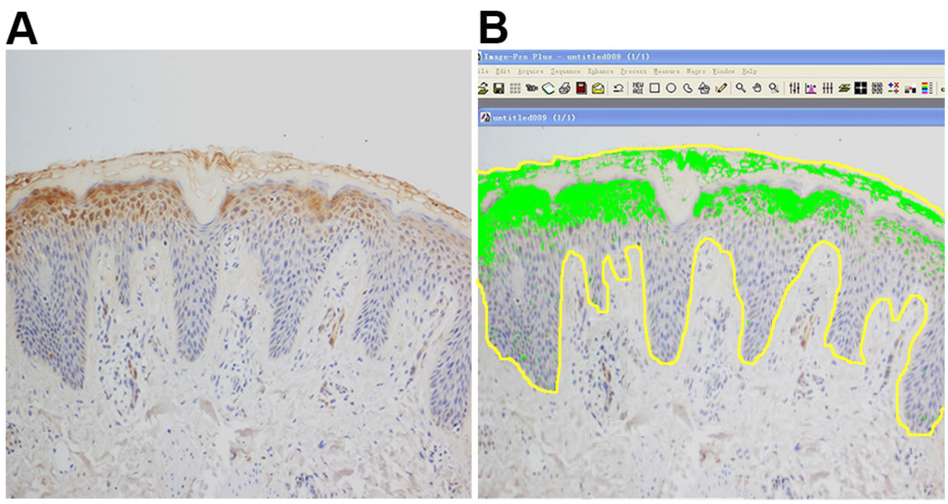

Figure 2.1 Quantification of staining intensity using Image-Pro Plus.
The images were recorded and saved digitally (A). The threshold of positive staining was defined using the eye-drop tool provided in the software. The epidermis was highlighted manually as an area of interest using the irregular tool. The optical counts of positive staining within AOI were measured based on intensity threshold (B).

2.2.4 *In situ* zymography

The activity of total epidermal proteases was examined by *in situ* zymography using casein-derived substrate. This casein derivative is extensively labelled with fluorogenic dye BODIPY TR-X, which results in the formation of intramolecularly quenched substrate and leads to a quenching of the fluorescent dye. Protease-catalyzed hydrolysis on the casein substrate releases the fluorescent dye-labelled peptides, allowing for the detection of protease activity. Briefly, the skin cryosections were air-dried and rinsed using 1x PBS with 0.1% Triton X-100 (Sigma-Aldrich, Poole, UK). Then the skin sections were incubated with 10 μg/ml BODIPY TR-X conjugated casein substrate (Life Technologies, Paisley, UK) in the developing buffer (10 mM Tris-HCl, pH=7.8) in a humid chamber at 37°C for two hours. After rinsing with PBS, nuclei were counter-stained with DAPI. Sections were again rinsed several times with PBS and mounted into Mowiol solution. The stained sections were kept at -20°C before being visualized using a Leica DMLB Microscope.

2.3 Construction of lentiviral vector and production of lentivirus

2.3.1 Construction of lentiviral vector

The full-length cDNA encoding human KLK5 was amplified by RT-PCR with forward primer (5'-GGGTGGTTATAACTCAGGCC-3'), and reverse primer (5'-GTGCATATCGCAGCAGTCGGTGG-3') (Invitrogen, Paisley, UK). The cDNA was cloned into pGEM-T vector (Promega, Hampshire, UK). Ligated vectors were transformed into E. coli competent cells JM109 (Promega, Hampshire, UK). Positive clones containing recombinant DNA were selected by white-blue screening using IPTG and X-gal (Sigma-Aldrich, Poole, UK). Presence of KLK5 gene insertion and the orientation of the insertion in the integrated vector were further confirmed using restriction digestion. The sequences of the recombinant pGEM-KLK5 plasmids were also checked using direct DNA sequencing (DNA sequencing services, Wolfson Institute for Biomedican Research, UCL), and the sequences were analyzed using Sequencher v5.2 (Gene Codes Corporation, USA).

Amplified KLK5 gene was further cloned to a HIV-1 based, self-inactivating lentiviral vector. The vector contains upstream spleen focus-forming virus (SFFV) promoter and downstream enhanced green fluorescent protein (eGFP) reporter gene. The vector encoding eGFP alone was used as negative control (Figure 2.2). SFFV promoter is known to mediate high levels of transgene expression in a diverse variety of target tissues. Lentiviral vector was digested using BamHI site, and pGEM-KLK5 vector was digested using SacI and SacII sites. All the restriction endonucleases used in this experiments were purchased from New England Biolabs (Hertfordshire, UK). Both digested lentiviral vector and KLK5 fragment were blunted. KLK5 gene was then cloned into the lentiviral vector using blunt end ligation to generate SFFV-KLK5-eGFP construct. Ligated vectors were transformed into E. coli competent cells. Clones with presence of KLK5 gene and correct orientation of insertion were selected using restriction digestion and direct DNA sequencing.

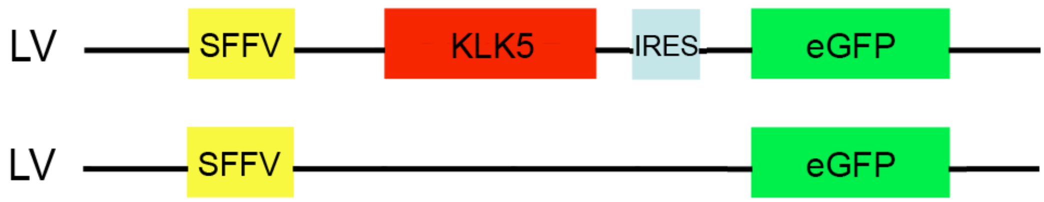

Figure 2.2 Construction of lentiviral vector.
KLK5 overexpression vector was constructed by cloning of KLK5 gene into a lentiviral vector containing upstream SFFV promoter and downstream eGFP reporter gene. KLK5 and eGFP genes were separated by an internal ribosome entry site (IRES). The vector containing eGFP only was used as control.

2.3.2 Production and titration of lentivirus

The 293T human renal epithelial cell line is a convenient source of recombinant virus. 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK) containing 200 mM L-glutamine, 10 mM MEM non-essential amino acids, 500 μg/ml geneticin and 100 mM sodium pyruvate, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in culture at 37^{o}C with 5% CO_2.

Lentivirus were produced by co-transfecting 293T cells with a packaging plasmid (pCMV8.74) encoding viral gag, pol and accessory proteins and an envelope plasmid (pMDG2) encoding the envelope of vesicular stomatitis virus. Twenty-four hours prior to transfection, 12 x 10^6 cells were seeded in each T175 flask. The following day, 293T cells cultured in T-175 flask were transfected with 25 μg of KLK5/eGFP or eGFP plasmid, 17.5 μg of pMDG2 plasmid and 25 μg of pCMV8.74 plasmid. Plasmid vectors and 1×10^{-7} mol/l of polyethylenimine (Sigma-Aldrich, Gillingham, UK) were diluted in OptiMEM (Invitrogen, Paisley, UK) and filtered through 0.22-μm pore cellulose acetate filters. Then the solution was gently mixed before being added to the cells.

Infectious lentiviruses were harvested 48 and 72 hours post-transfection. The culture supernatants were collected and centrifuged at 2,000 R.P.M. for 5 minutes to get rid of the cell debris, followed by being filtered through 0.45-μm pore filters. The filtered supernatants were concentrated by ultracentrifugation at 23,000 R.P.M for 2 hours. The final viral pellets were resuspended in OptiMEM for 30 minutes on ice and aliquots of viral stocks were kept at −80 °C until further use.

For the titration of lentivirus, $1x10^5$ of 293T cells were seeded on 24 wells plate and infected with serial dilutions of KLK5/eGFP or eGFP lentiviral stock. Multiplicity of infection (MOI) used for the lentiviral infection ranged from 10 to 0.0032. Transduced cells were identified by eGFP fluorescence using flow cytometry. Briefly, a suspension of non-fixed cells was analyzed on a BD LSRII FACS machine (BD, San Jose, CA, USA). Forward and side scatter were used to exclude debris and aggregates, and eGFP fluorescence was measured by filter 530/30 nm. Transduced cells were identified by eGFP fluorescence. For each sample, 10,000 eGFP positive cells were collected. Analysis was performed using FlowJo (Tree Star, Stanford University, San Francisco, CA, USA). Transducing activity was expressed in transducing units (TU). The titers of the virus in transducing units per ml (TU/mL) was determined using the following formula: virus titers = [(cells at starting time) x (percentage of transduced cells)]/(volume of virus solution added expressed in mLs). Titres of eGFP and KLK5/eGFP lentivirus were $8x10^8$ and $4x10^8$ TU/mL respectively.

2.4 Human keratinocytes culture and lentiviral transduction

2.4.1 Culture of human keratinocytes

Human keratinocytes were co-cultured with lethally irradiated 3T3 mouse fibroblasts and grown in the keratinocyte culture media. The culture media was consisted of equal amount of DMEM and DMEM/Ham F12 (DMEM/F12, 3:1 mixture) (Invitrogen, Paisley, UK) supplemented with 10% FCS and Pen/Strep (100 IU/ml of penicillin, 100 μg/ml of streptomycin). Human keratinocyte growth supplement (Invitrogen, Paisley, UK) was added to the culture media, and the final concentrations of the components in the supplemented medium were: 0.2% bovine pituitary extract, 5 μg⁄ml bovine insulin, 0.18 μg⁄ml hydrocortisone, 5 μg⁄ml bovine transferrin, 0.2 ng⁄ml human epidermal growth factor. Keratinocytes were maintained in culture at 37^oC, 5% CO_2 and passaged every 3-4 days at subconfluence and fresh 3T3 feeder cells were added when needed.

Three types of human keratinocytes were used: NIKS, NTERT and primary human keratinocytes. NIKS is a spontaneously immortalized human keratinocyte cell line

(Allen-Hoffmann et al. 2000). NTERT cells are immortalized human keratinocytes by ectopically expressing of the telomerase catalytic subunit hTERT (Dickson et al. 2000). Both cell lines become immortal but still retain normal growth and differentiation characteristics. As cell lines are easy to be handled and cultured, they are important tools for the *in vitro* study. However primary cell cultures mimic the physiological state of cells more closely and are more suitable for the *in vitro* and *in vivo* function study. Therefore, primary human keratinocytes were also used in this study.

In order to isolate human primary keratinocytes, sterile skin biopsies were obtained by punch biopsy from healthy volunteer donors or AD patients. Each individual has been given written informed consent initially when the biopsy was taken. The biopsies were then washed in PBS and excess dermis together with underneath connective tissues were removed using sterile blade. The skin samples were cut into small pieces and immersed in the Dispase solution (50U/mL, BD Biosciences, Plymouth, UK) at $4^{o}C$ overnight. Next day, the epidermis was mechanically separated from the dermis using forceps after the enzymatic treatment with Dispase. The epidermis was cut into small pieces and incubated with 0.25% trypsin/EDTA at $37^{o}C$ for 40 minutes with agitating every 10 minutes. Equal volumes of 10% FCS/DMEM were then added into the digested contents to neutralise the trypsin. The contents were filtered through sterile 100µM cell strainer (BD Biosciences, Plymouth, UK) and the filtered suspension was centrifuged at 2,000 R.P.M. for 10 minutes. The cell pellet was resuspended in keratinocyte culture media and seeded in the flask with 3T3 feeder layer.

2.4.2 Lentiviral transduction of human keratinocytes

For lentiviral transduction, $2x10^{5}$ of human keratinocytes were seeded on 12 wells plate 24 hours prior to transduction. On the following day, cells were infected with KLK5/eGFP or eGFP lentiviral stock without addition of a cationic polymer. Following exposure to one round of lentiviral infection at MOI 10, transduced cells with eGFP fluorescence were visualized under Leica DM IL inverted fluorescent microscope and transduction efficiencies were assessed at different time points. Percentage of GFP positive cells in transduced keratinocytes was also evaluated using flow cytometry. Briefly, cultured cells were trypsinized and resuspended in PBS with 5% FCS. The suspension of non-fixed cells was analyzed on a BD LSRII FACS machine (BD, San Jose´, CA, USA). Forward and side scatter were used to exclude debris and aggregates, and eGFP fluorescence was measured by filter 530/30 nm. Transduced cells were identified by eGFP fluorescence. In all cases, 10,000 eGFP positive cells were collected. Analysis was performed using FlowJo (Tree Star, Stanford University, San Francisco, CA, USA).

2.5 Immunocytostaining

Over twenty-four 13mm glass coverslips were immersed in 70% alcohol (IMS) in a sterile petri dish for approximately ten minutes. Then the coverslips were taken out from the petri dish and left to dry in the hood. Each coverslip was transferred into an individual well of a 24-well plate. $2x10^5$ cells were seeded in each well and the plate was stored in an incubator at 37^oC, 10% CO_2 for 2 days. Prior to fixation, all cells were washed twice with PBS. For fixation and permeablilization of cells, the coverslips were immersed in 4% paraformaldehyde solution with 0.1% Triton X-100 for 10 minutes, after which the coverslips were transferred into 35mm petri dishes. Then the cells were washed three times using PBS. After the third wash, the cells were left in PBS and stored at 4°C before staining.

Immunofluorescence staining was performed by adding 30μl of diluted anti-KLK5 antibody to each coverslips, proceeded by immediate coverage using a small piece of parafilm. Then the cells were incubated with the primary antibody in a humid chamber at 4°C overnight. Negative controls were included for each sample by omitting the primary antibody. Following by three repeated washes with PBS, each coverslip was incubated with Alexa Fluor 568–coupled goat anti-mouse IgG in dark at room temperature for one hour. The cells were then washed three times in PBS and left in the final wash for 15 minutes. Nucleus was counterstained with DAPI, followed by further washing of the cells twice in PBS. The coverslips were mounted onto glass slides using Mowiol solution (Calbiochem, Nottingham, U.K.) and stored at -20°C. Images were recorded using a LSM 510 UV confocal laser scanning microscopy (Zeiss, Germany) and processed using ZEN 2009 Light Edition (Zeiss, Germany).

2.6 Western blotting

2.6.1 Protein assay

The cell pellets were resuspended in ice-cold lysis buffer, pH 8.0. The final concentrations of the components in the lysis buffer were: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1x protease inhibitor cocktail (Roche, Mannheim, Germany), 1mmol/L phenylmethanesulfonylfluoride and 1% Triton. Samples were incubated on ice for 15 minutes. To lyse the cells completely, the solution was pipetted up and down for several times, prior to being centrifuged at 12,000 R.P.M. for 15 minutes at 4oC to pellet the insoluble material. The total protein concentration in both cell lysates and culture supernatants were determined using Bio-Rad protein assay kit (Hertfordshire, UK). Two-fold serial dilutions of protein standard bovine serum albumin (BSA) that cover the range from 3.125 μg/ml to 50 μg/ml were used to generate the standard curve. Serial dilutions of both the protein supernatant and BSA protein were then prepared, after which the absorbance of each sample was read at a wavelength of 595nm. The concentration of total protein was calculated in reference to serial dilutions of BSA falling within the linear part of the standard curve.

2.6.2 SDS-PAGE and protein transfer

The cell lysates or culture supernatants were diluted in 5x sample buffer containing 0.5mmol/l Tris-HCl, 100mmol/l dithiothreitol, 10% sodium dodecyl sulfate (SDS), 30% glycerol and 0.001% bromophenol blue, pH 6.8. The samples were heated at 95^{o}C for 5 minutes. The gels were prepared according to the recipe (Table 2.1). Once the gels were prepared, 5μl of full range Rainbow molecular weight marker (GE Healthcare, Buckinghamshire, UK) was loaded. Equal amount of total protein in appropriate volumes of each sample were also loaded in sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were then run at 100V in running buffer for approximately three hours, until the samples had run to the end of the gel. The components of the running buffer were: 25 mmol/L Tris-base, 190 mmol/L glysine and 0.1% SDS in dH_2O.

	Company	8%	10%	12%	Stacking gel
H_2O	-	4.71 mL	4.05 mL	3.35 mL	3.05 mL
1.5 M Tris-HCl, pH 8.8	Sigma	2.5 mL	2.5 mL	2.5 mL	-
0.5 M Tris-HCl, pH 6.8	Sigma	-	-	-	1.25 mL
30% Acrylamide/Bis-acrylamide	Sigma	2.64 mL	3.3 mL	4 mL	0.65 mL
20% (w/v) SDS	Sigma	100 μl	100 μl	100 μl	50 μl
10% ammonium persulfate (APS)	VWR	150 μl	150 μl	150 μl	37.5 μl
TEMED	Sigma	30 μl	15 μl	15 μl	7.5 μl

Table 2.1: Recipe of the separating and stacking gels for SDS-PAGE.

After electrophoresis, proteins were transferred to Hybond-P polyvinylidene fluoride membranes (GE Healthcare, Buckinghamshire, UK) at 100V for 1 hour and 30 minutes.

Prior to transfer, a nitrocellulose membrane, several pieces of filter paper and sponges were all soaked in transfer buffer for at least 15 minutes. The stacking gel was removed and the remaining gel transferred to the saturated membrane stacked on top of the moist sponge and filter paper. The gel was covered with a second piece of soaked filter paper and sponge, all of which were contained within a plastic enclosed grid and transferred into the transfer chamber filled with transfer buffer. The components of the transfer buffer were: 25 mmol/L Tris-base, 190 mmol/L glysine, 20% methanol in dH_2O. The chamber was kept within an ice bucket.

2.6.3 Detection

The membrane was washed for 3 times by soaking in wash buffer that contains PBS with 0.05% Tween 20 on a shaker. After washing, the membrane was blocked with block solution containing 5% dried skimmed milk in wash buffer for 1 hour. This was followed by three washes, each for 5 minutes in wash buffer. The membrane was then incubated with primary antibodies in a sealed plastic bag on a vigorous shaker overnight. All the primary antibodies were diluted in 3% BSA in PBS. Information of the primary antibodies used for western blot analysis was listed in Table 2.1 (section 2.17). On the next day, the membranes were washed by 3 times and further incubated with sheep anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (HRP) (GE Healthcare, Buckinghamshire, UK) or mouse anti-rabbit immunoglobulin G antibody conjugated with HRP (Sigma-Aldrich, Gillingham, UK) in a sealed bag for 1 hour. The secondary antibodies were diluted in 3% dried skimmed milk in wash buffer. This was followed by three consecutive washes in wash buffer each for 10-15 minutes. Signals were detected using the ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) following by the instruction of the manufacturer. All experiments were performed in duplicate, and densitometry analysis was carried out on scanned immunoblot images.

2.7 Casein gel zymography

NIKS and NTERT cells were cultured in serum free keratinocyte culture media for 48 hours. The culture media were collected and concentrated using Amicon centrifugal filter devices (Millipore, Watford, UK). Concentrated samples were mixed in a non-reducing Novex® Tris-Glycine SDS Sample Buffer (Life Technologies, Paisley, UK). And then the samples were loaded onto polyacrylamide gels copolymerized with casein substrate (12% acrylamide, 0.1% casein, Life Technologies, Paisley, UK) for electrophoresis. After electrophoresis, the gels were soaked in renaturing buffer containing 50 mM Tris, pH 8 and 2.5% Triton X-100 (Life Technologies, Paisley, UK) for 1 hour at room temperature to remove SDS. Then the gels were incubated at 37°C in developing buffer containing 50 mM Tris, pH 8 (Life Technologies, Paisley, UK) for 24 hours. Gels were stained with 1% Coomassie Brilliant blue (Sigma-Aldrich, Poole, UK) for 1 hour. Casein degrading activity could be visualized when the gels were stained with Coomassie Blue. Areas with caseinolytic activity showed as clear bands against the dark blue background, revealing the digestion of substrate on the gel.

2.8 Intracellular calcium mobilization assay

Measurement of intracellular [Ca^{2+}] mobilization was performed using the FluoForte Calcium Assay kit (Enzo Life Sciences, Exeter, UK). Mobilization of intracellular calcium was detected utilizing a fluorogenic calcium-binding dye. The dye is provided to the cells as an acetoxymethyl ester, which is cell-permeable. Once inside the cells, the dye is hydrolyzed by intracellular esterases, leading to the formation of a cell membrane impermeable form of this dye. In the absence of calcium, the calcium-binding moiety portion of the dye quenches the fluorescence of its fluorophore portion. The self-quenching of this dye was relieved upon binding of mobilized calcium, resulting in an increased fluorescence signal.

Human keratinocytes were plated in 96-well plates at the density of 1×10^4 cells/100 µl per well. After 24 hours, the growth medium was completely removed. Dye-loading solution was prepared by reconstituting one vial of Reagent A in assay buffer containing 1mL of Reagent B and 9mL of Reagent C, and 100 µl of dye-loading solution was added per well. The cells were further incubated with the dye-loading solution for 45 min at 37°C and then 15 min at room temperature. The cells were stimulated with 25µM of recombinant human KLK5 (rKLK5) from commercial source (R&D Systems, Oxfordshire, UK) or 100µM of PAR2 activating peptide (SLIGKV-NH2, H-5024, Bachem, Switzerland), and a time-response curve of intracellular [Ca^{2+}] signal was recorded via real-time monitoring of fluorescence intensity at excitation = 530 nm and emission = 570 nm in a microplate reader (FLUOstar, OPTIMA). For the inhibitory study, during the incubation of the cells, concentrated culture media of KLK5-cells containing recombinant KLK5 was pre-incubated with SFTI-G at different concentrations at 37°C for 10 minutes. Normal keratinocytes were challenged with the mixtures of pre-incubated KLK5 and SFTI-G. Cells injected with KLK5 alone were used as control. The response curve of intracellular [Ca^{2+}] mobilization was recorded.

Intracellular calcium mobilization was shown as an increase of fluorescence intensity in relative fluorescence units (RFU), and the RFU values were plotted against the time.

2.9 Three-dimensional organotypic cultures (OTCs)

Following a single passage, primary keratinocytes were cultivated *in vitro* on de-epidermalized dermal (DED) matrix. Briefly, glycerol-preserved DED matrix (Euro Skin Bank, Beverwijk, Netherlands) was incubated in PBS containing antibiotic mix (600 units/mL penicillin-G, 600 µg/mL streptomycin sulfate, 250 µg/mL gentamicin sulfate, and 2.5 µg/mL fungizone) at 37°C for up to 10 days and then cut into 1.5 × 1.5 cm^2. Dermal fibroblasts derived from normal human skin biopsies (passages 3–7) were used for the culture. Primary human fibroblasts were seeded onto the reticular side of the DED matrix overnight. Next day, the keratinocytes were seeded on the papillary side of the DED matrix (Figure 2.3).

After keratinocytes reached confluence (about 2–3 days), the culture was lifted and then cultivated at the air-liquid-interface for 14 days to allow keratinocyte differentiation. The mature cultures were fixed in 4% paraformaldehyde at 4°C overnight, following by being embedded in paraffin or snap frozen in liquid nitrogen in Tissue-Tek O.C.T. All paraffin-embedded or snap frozen tissues were cut into skin sections (5µm thickness) and collected onto glass slides. For histological examination, paraffin-embedded sections were stained with hematoxylin and eosin. Immunostaining for KLK5, FLG and DSG1 was performed on paraffin-embedded sections or cryosections.

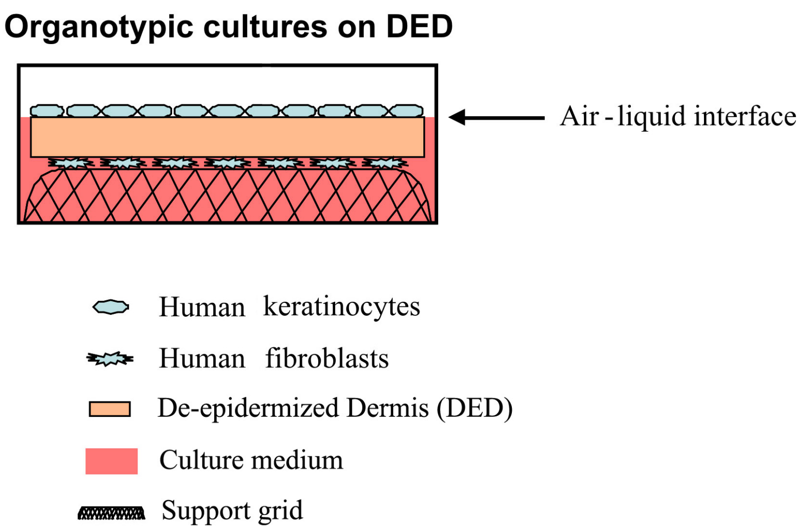

Figure 2.3: In vitro assessment using three dimensional organotypic cultures.
Primary keratinocytes were cultured in vitro on DED matrix at the air–liquid interface for 14 days (Adapted from Martins et al. 2009).

2.10 Bioengineered skin preparation and grafting to immunodeficient mice

Primary normal human keratinocytes infected with lentiviral vectors encoding eGFP or KLK5/eGFP genes were seeded on top of a fibrin matrix populated with live primary human fibroblasts (Figure 2.4 A). After keratinocytes reached confluence, the bioengineered skin constructs were grafted onto the dorsum of six-weeks old female immunodeficient nude/nude mice (NMRI strain, Elevage-Janvier, France) (Figure 2.4 B). Eight weeks after grafting, skin samples from grafts were taken postmortem, embedded in paraffin or snap frozen and sectioned for histological and immunohistochemical examination.

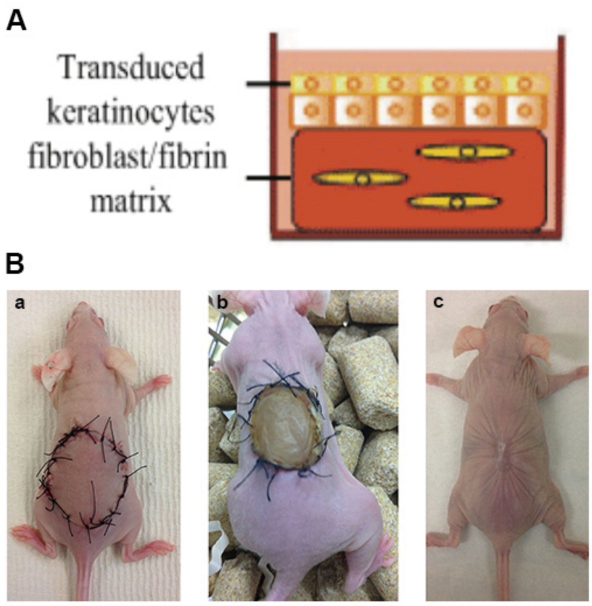

Figure 2.4: In vivo evaluation using a humanized mouse model.
(A) Formation of the skin-equivalent constructs (Di Nunzio et al. 2008).
(B) Keratinocytes were grafted onto nude mice based on two independently conducted experiments (a), and scar was formed and peeled off about 2 weeks post-grafting (b). Regenerated skin grafts were examined 8 weeks after grafting (c).

2.11 RT^2 profiler Human EGF PCR array

The SYBR-Green fluorescence-based human EGF PCR array (Qiagen, Manchester, UK) was used to evaluate the expression of 82 genes related to EGF-mediated signal transduction in keratinocytes transduced with KLK5/eGFP or eGFP only vector. The experiment was conducted by following the instruction of manufacturer. Total RNA was extracted using Trizol reagent (Sigma-Aldrich, Gillingham, UK). cDNA was synthesized using the RT^2 First Strand Kit according to the protocol provided by the manufacturer. cDNA template was mixed with the appropriate ready-to-use PCR master mix, and equal volumes were aliquot to each well of the same plate. Then the plate was run using the real-time PCR cycling program on a real-time thermal cycler (7300, Applied Biosystems). PCR conditions consist pre-heating of the thermal sensitive HotStart DNA polymerase at 94ºC for 10 minutes, followed by 40 cycles of PCR performed at 95ºC for 15 seconds, 60ºC for 1 minute.The expression of target genes was normalized to the housekeeping genes. Fold change was calculated as the normalized gene expression (2^(- Delta Ct)) in KLK5-cells divided by the normalized gene expression in eGFP-cells. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicates a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change. Three-fold change was used as the threshold.

2.12 Reverse transcription-PCR (RT-PCR)

Total RNA was isolated from samples using Trizol reagent (Sigma-Aldrich, Gillingham, UK). cDNA was synthesized from RNA template using first strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). The reverse transcription reaction was performed using 1µg RNA diluted in 1µL nuclease free water, 2µL first strand buffer, 4µL MgCl2 solution, 1µL random primers, 8µL dNTPs, 1µL RNase inhibitor and 1µL reverse transcriptase. The sample was incubated at room temperature for 30 minutes. After that, 1 cycle of PCR was performed at 42ºC for 45 mins, 99ºC for 5 mins and 5ºC for 5 mins in a PCR cycler. The expression of KLK5 and GSK-3b was determined using PCR. A 25 µL reaction was performed using 100ng of cDNA template, 12.5µL of PCR mastermix, 2.5µL of upstream primer, 2.5µL of downstream primer, 0.625U of Taq polymerase and nuclease free water.

All the primers were purchased from Invitrogen, and primer sequences were as follows:KLK5: forward primer (5'-GGGTGGTTATAACTCAGGCC-3'); and reverse primer (5'-GTGCATATCGCAGCAGTCGGTGG-3'); GSK-3b: forward primer (5'CAGTTTTCCTGGCCAGGAGC-3'); and reverse primer (5'-CCCAAAGAGGTAAGCGCTAC-3'). PCR conditions consist pre-heating of the thermal sensitive Taq polymerase at 94 ºC for 2 minutes, followed by 40 cycles of PCR performed at 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds and 72 ºC for 10 minutes in a PCR cycler. The expression of KLK5 and GSK-3b was normalized to the housekeeping gene GAPDH.

2.13 Human phospho-kinase antibody array

The Human Phospho-Kinase Antibody Array (R&D Systems, Oxfordshire, UK) was used to probe 43 phospho-kinases and 2 related total proteins heat shock protein 60 (HSP60) and β-catenin in the cell lysates of cultured keratinocytes. The experiment was conducted by following the procedures recommended by manufacturer. Briefly, the array membranes were first incubated in the block buffer for 1 hour. In the meantime, cells were lysed in the by incubating in the lysis buffer on ice for 15 minutes. The samples were then centrifuged at 12,000 R.P.M. for 15 minutes to pellet the insoluble material. The total protein concentration in the cell lysate was determined using Bio-Rad protein assay kit (Hertfordshire, UK). Desired quantity of cell lysates were diluted with the blocking buffer. After being removed from the block buffer, the array membranes were incubated with the diluted cell lysates at 4 ºC overnight. After incubation, the membranes were washed 3 times with wash buffer containing PBS and 0.05% Tween 20 for 5 minutes each and incubated with the detection antibody cocktail at room temperature for 1 hour. Following incubation, the membranes were washed with 3 changes of wash buffer that contains PBS and 0.05% Tween 20 for 5 minutes each and then incubated with the steptavidin-HRP solution at room temperature for 30 minutes. The membranes were finally washed with wash buffer for 3 times at room temperature and the bound antibodies were detected by chemiluminescence using the reagents provided by manufacturer. The intensities of the blots were quantified by densitometry. As each reference or target protein was blotted in duplicate, mean pixel density of each pair of duplicate blots was calculated as a result of the protein level of each reference or target.

2.14 Human cytokine antibody array

The Human Cytokine Antibody Array Panel A (R&D System, Oxfordshire, UK) was used to probe 36 cytokines in the culture supernatants collected from keratinocytes. The experiment was conducted by following the procedures recommended by manufacturer. Briefly, the array membranes were first incubated in the block buffer for 1 hour. In the meantime, desired quantity of culture media was mixed with the detection antibody cocktail and incubated at room temperature for 1 hour. After removing the block buffer, the sample/antibody mixture were added to the array membranes and incubated at 4 ºC overnight. Followed by the incubation, the membranes were washed 3 times with wash buffer that contains PBS and 0.05% Tween 20 for 5 minutes each and then incubated the steptavidin-HRP solution at room temperature for 30 minutes. The membranes were finally washed with wash buffer for 3 times at room temperature and the bound antibodies were detected by chemiluminescence using the reagents provided by manufacturer. The intensities of the blots were quantified by densitometry. As each reference or target protein was blotted in duplicate, mean pixel density of each pair of duplicate blots was calculated as a result of the protein level of each reference or target.

2.15 Enzyme linked immunosorbent assay (ELISA)

Culture supernatants were collected 48 hours after the cells reached confluence, followed by centrifugation at 1,200 R.P.M for 7 min at 4°C to remove the cellular debris. The total protein concentration in culture supernatant was quantified using Bio-Rad protein assay kit (Section 2.5.1). The samples were then stored at −80°C until being subjected to ELISA. Expression of IL-8 in the culture supernatants was measured by ELISA using mouse monoclonal antibody to human IL-8 (BD Biosciences, San Diego, CA, USA). Immunoreactive TSLP and IL-10 within the supernatants were also quantified using ELISA kits containing mouse monoclonal antibodies against human TSLP and IL-10 (eBioscience, Hatfield, UK). These antibodies had no measurable cross reactivity with high concentrations of other known cytokines as claimed by the manufactures. Serial dilutions of recombinant standards and samples were carried out according to the instructions provided by the manufacturers. The standard curves were generated using the recombinant standards within certain range. The expression of protein was quantified in reference to serial dilutions of recombinant standards falling within the linear part of the standard curve. The levels of IL-8, TSLP and IL-10 were normalized to the concentration of total protein in each sample. Data represent the results from three independent experiments performed in duplicate.

2.16 Statistical analysis

Each experiment was performed independently for two or three times. Data were expressed as mean ± standard error of the mean (SEM) of one experiment performed in duplicate or triplicate. The appropriate statistical tool was used as described in each chapter. For comparison of protein expression in normal, AD nonlesional and AD lesional skin, data were analyzed statistically using one-way ANOVA with post-hoc Tukey tests. The levels of different proteins in untransduced cells, eGFP-cells and KLK5-cells were compared by one-way ANOVA with post-hoc Tukey tests. For comparison of SFTI-G treated and untreated groups, student's t-test was used to determine the statistically significant differences between the treated and untreated groups. Statistical evaluation of data was performed using SPSS 16.0 (SPSS Inc., Chicago, USA). Differences with p-value less than 0.05 were considered as statistically significant.

2.17 Antibodies

The information and dilutions of the antibodies used in immunostaining and western blotting were listed in Table 2.2.

Antibody	**Raised species**	**Clone**	**Product code**	**Company**	**Dilution for staining**	**Dilution for western blotting**
KLK5	mouse	-	H000258 18-B01P	Abnova, Taiwan, China	1:500	1:5000
DSG1 (extracellular)	mouse	P124	10R-D104A	Fitzgerald, Cambridge, UK	1:100	1:100
PAR2	mouse	Sam11	sc-13504	Santa Cruz, Heidelberg, Germany	1:50	1:200
FLG	mouse	15C10	NCL-FILAGG RIN	Leica Microsystem, Newcastle, UK	1:100	-
Involucrin	Mouse	SY5	19018	Sigma-aldrich, St. Louis, USA	1:10000	-
GSK-3β	rabbit	Y174	ab32391	Abcam, Cambridge, UK	-	1:5000
Phospho-p53	rabbit	-	AF2996	R&D, Oxfordshire, UK	-	1:2000
HSP60	mouse	264233	MAB 1800	R&D, Oxfordshire, UK	-	1:1000
Actin	mouse	AC-15	A5441	Sigma-aldrich, St. Louis, USA	-	1:10000

Table 2.2 List of antibodies used for immunostaining and western blotting.

CHAPTER 3: RESULTS

3.1 Disturbed epidermal barrier in AD

Epidermal barrier dysfunction is considered as the initial event in AD. Various genetic and environmental factors can contribute to enhanced activity of epidermal proteases, consequently exacerbating the skin barrier defect and promoting the development of AD. KLK5 is one of the key epidermal proteases accounting for most of the trypsin-like proteolytic activity (Brattsand et al. 2005). It is also involved in the regulation of epidermal barrier function through DSG1, PAR2 and ELA2 (Caubet et al. 2004; Descargues et al. 2006; Briot el al. 2009; Bonnart et al. 2010). It was speculated that up-regulation of KLK5 might play an important role in skin barrier dysfunction in AD. Although previous reports showed increased expression and elevated proteolytic activity of KLK5 in AD lesions (Komatsu et al. 2005, 2007b; Voegeli et al. 2009), there were few studies evaluating the expression of KLK5 protein in skin samples obtained from a group of AD patients by comparing to a group of age-matched healthy donors. In this study, the epidermal morphology, the expression of KLK5 and the levels of relevant barrier-related proteins were examined in the AD and normal skin. Skin biopsies were taken from the nonlesional and lesional sites of five AD patients and five age-matched healthy donors (Section 2.2.1).

3.1.1 Impaired epidermal morphology in the AD skin

The epidermal morphology in AD and normal skin was examined by H&E staining (Section 2.2.1). The lesional skin from all five AD patients exhibited morphological changes by comparing to the normal skin (Figure 3.1, k-o vs. a-e). Histopathological features of AD lesion consisted of acanthosis (thickening of the epidermis), parakeratosis (retention of nucleus in the cornified layer), elongated rete ridges of the epidermis and superficial dermal perivascular inflammatory infiltrate. In contrast, the conified layer remained relatively intact in the nonlesional skin compared to the lesional skin. However, the nonlesional skin already exhibited some AD-like histopathological characteristics (Figure 3.1, f-j), but far less prominent than those observed in the lesional skin.

In addition, keratinocytes were tightly connected in the normal skin under higher magnification, whereas enlarged intercellular space between neighboring keratinocytes was observed in the AD lesional skin, as well as the nonlesional sites of some AD patients (Figure 3.2).

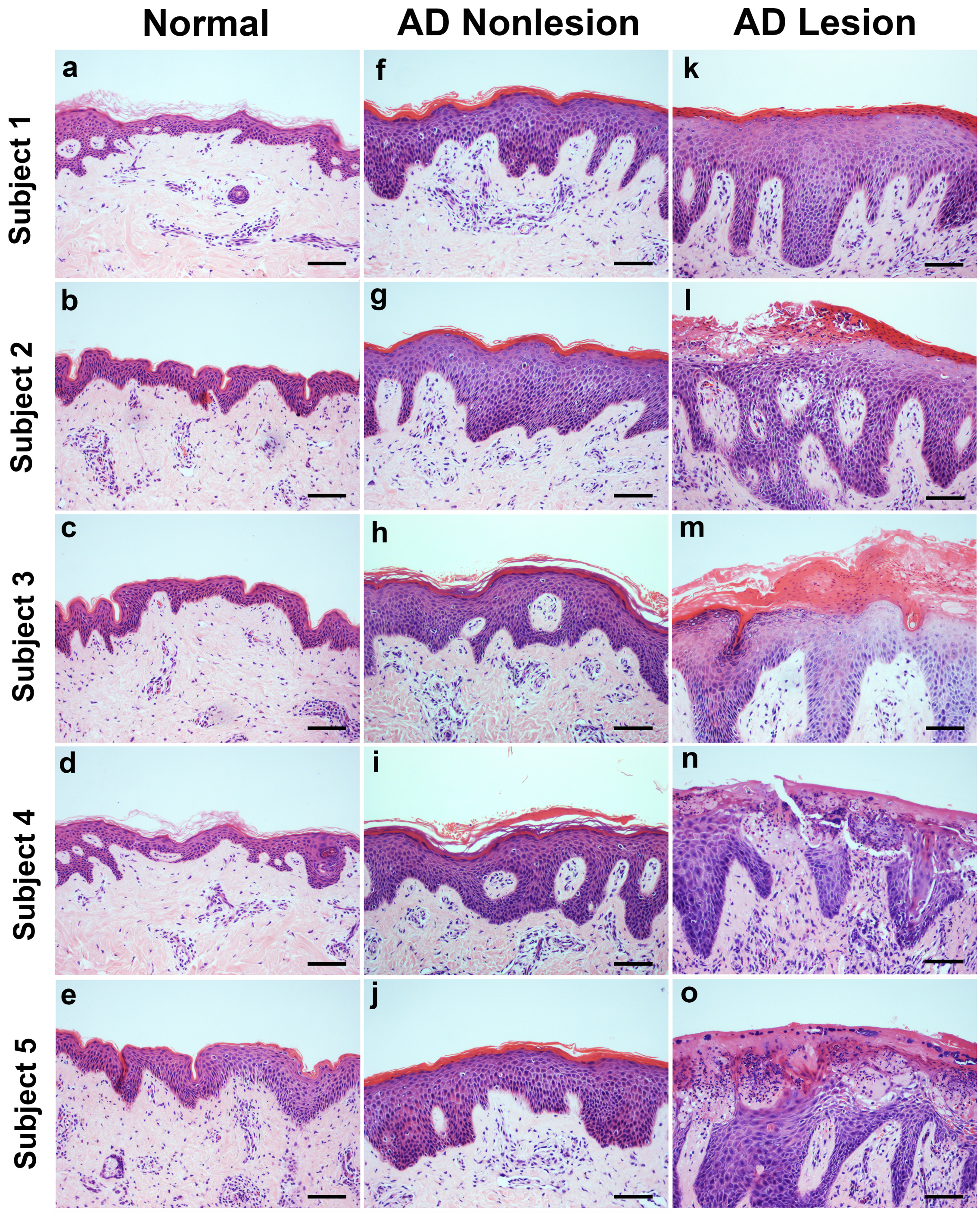

Figure 3.1. Impaired epidermal barrier in the AD skin.

H&E staining was performed on the normal and AD skin. The AD lesional skin exhibited hyperplasia, parakeratosis, elongated rete ridges and lymphocyte infiltration compared to the normal skin (k-o vs. a-e). The nonlesional skin also showed thickened epidermis and parakeratosis, but not as obvious as those in the lesional skin (f-j). Bar= 100µm.

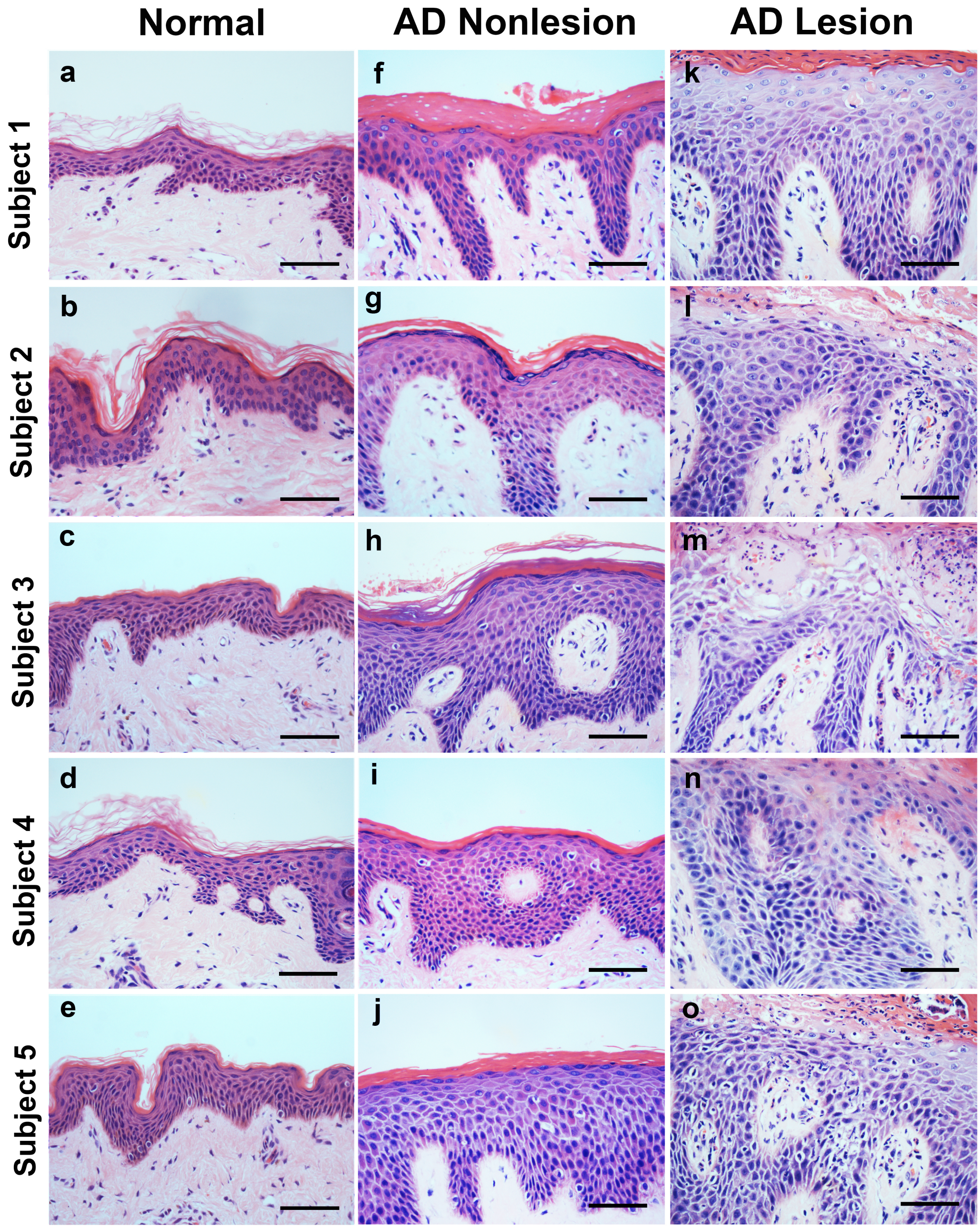

Figure 3.2. Enlarged intercellular space in the AD skin.
H&E stained sections were further checked under microscope at higher magnification. Enlarged intercellular space was observed in the AD lesional skin (k-o) as well as the nonlesional skin from three subjects (h-j) compared to the normal skin (a-e). Bar= 50 µm.

3.1.2 Up-regulation of KLK5 in AD

The expression of KLK5 was examined in the normal and AD skin by immunohistochemistry staining using anti-human KLK5 antibody (Section 2.2.2).

The results showed that KLK5 was mainly localised in lower cornified layer of the epidermis in normal skin, whereas the staining of KLK5 was extended to deeper layers with increased intensity in the AD lesional skin (Figure 3.3, a-e vs. k-o). Enlarged staining zone and enhanced staining intensity of KLK5 were already observed in the nonlesional sites of some AD patients (Figure 3.3, h and j), but not as prominent as those detected in the lesional skin.

The intensity of KLK5 staining was quantified using computer-assisted image analysis (Section 2.2.3). The results indicated that there was significantly elevated expression of KLK5 in the AD lesional skin compared to the normal skin ($p<0.05$) (Figure 3.4). In contrast, the increase of KLK5 staining in the nonlesional skin was not significant ($p>0.05$). These results revealed up-regulated expression of KLK5 in AD skin especially in the lesional sites.

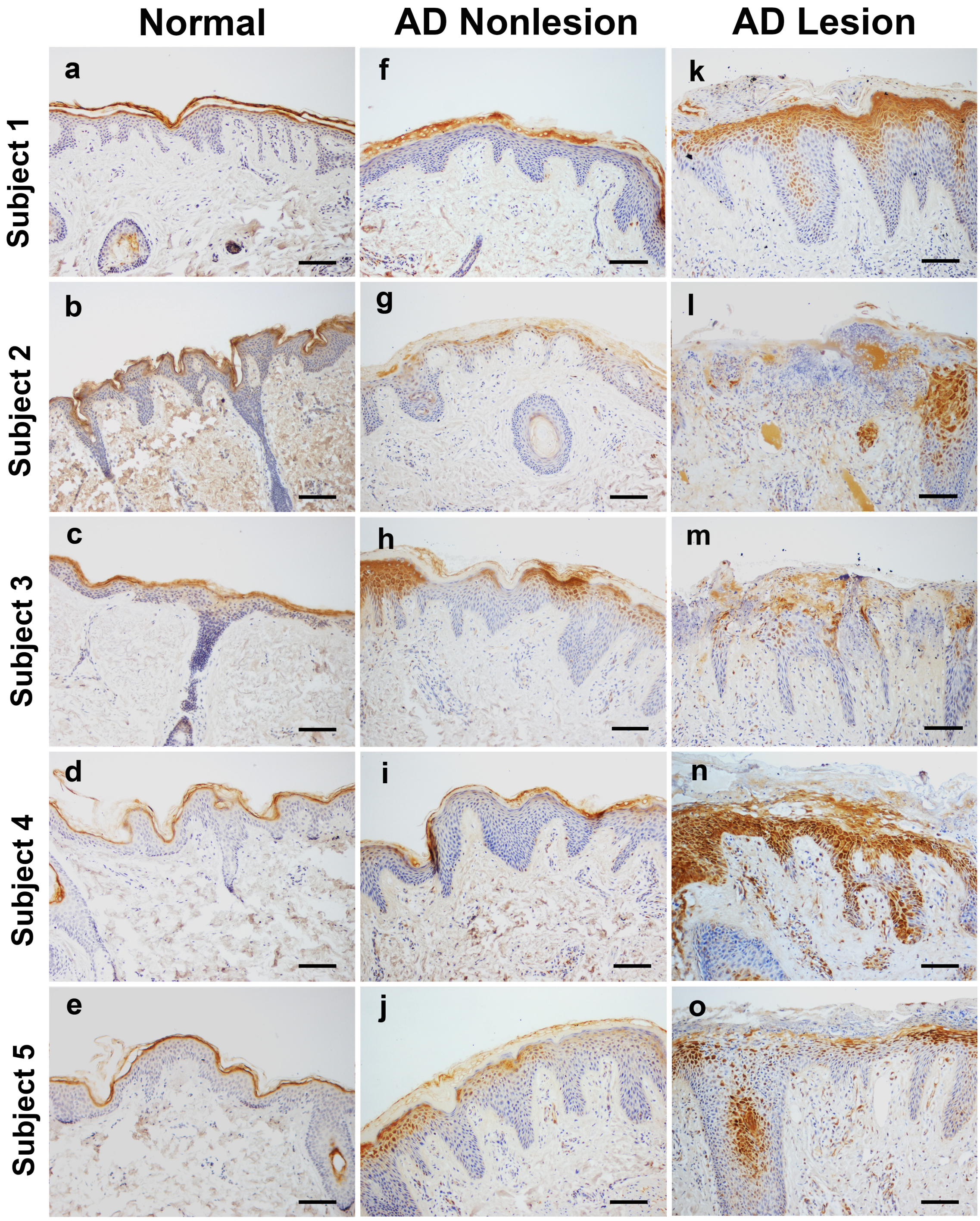

Figure 3.3. Aberrant expression of KLK5 in the AD skin.

Immunohistochemistry staining of KLK5 was carried out in the normal and AD skin. Brown color represents the staining of KLK5. Blue color represents hematoxylin-stained nuclei. Extended staining of KLK5 in the deeper layers of the epidermis with increased intensity was found in the lesional skin (k-o) compared to the normal skin (f-j). There was also extended expression of KLK5 in the nonlesional skin, but not as prominent as the lesional skin. Bar= 100 µm.

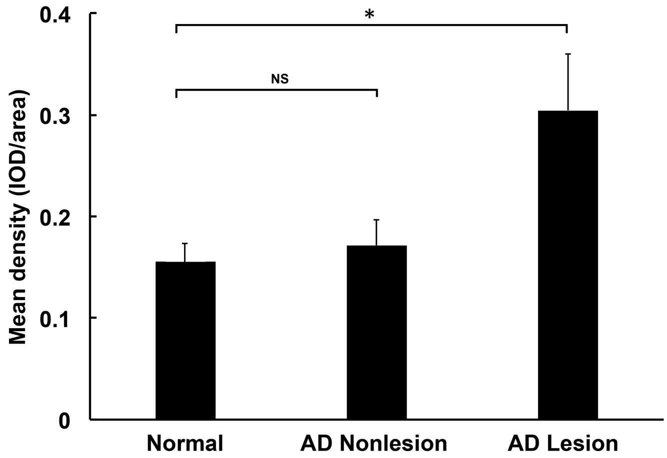

Figure 3.4. Quantification of KLK5 expression in normal and AD skin.
*The expression of KLK5 was quantified according to the intensity of KLK5 staining in the skin biopsies. The images of three non-overlapped but adjacent regions in each skin section were recorded and saved digitally. The epidermis in each region was highlighted manually as AOI. The staining intensity in each AOI was shown as mean density, which was calculated as integrated optical density (IOD)/area. The results showed significantly increased expression of KLK5 in the AD lesional skin (n=5), while there was no significant difference in KLK5 expression between nonlesional skin (n=5) and normal skin (n=5). Error bars represent the standard error. * indicates results significant at p<0.05.*

3.1.3 Up-regulated activity of epidermal proteases in the AD skin

KLK5 is a major contributor to trypsin-like proteolytic activity and the initiator of protease activation cascade in the epidermis. A study from another research group has showed increased activity of the extractable trpsin-like proteases in the tape strips obtained from AD lesions, which could be attributed to unregulated activity of KLK5 (Voegeli et al. 2009). Although the staining results in our study showed up-regulated expression of KLK5 in the AD skin, the activity of KLK5 in AD still needed to be further investigated. However, there is no *in situ* approach available to evaluated the proteolytic activity of KLK5 in the skin at present. Therefore, the activity of total epidermal proteases was examined by *in situ* zymography using the casein-derived substrate labelled with fluorogenic dye. Protease-catalyzed hydrolysis on the substrate releases the fluorescent dye-labelled peptides, allowing for the detection of protease activity (Section 2.2.4). As KLK5 is one of the main contributors to epidermal proteolytic activity, the results from this assay could partially represent the activity of KLK5.

The results showed that the epidermal proteolytic activity was low and mainly restricted to the cornified layer in the normal skin. In contrast, the activity was extended from the cornified layer to the deeper layers in the AD lesional skin with increased intensity, indicating enhanced activity of epidermal proteases in AD (Figure 3.5 i-l vs. a-d). In the nonlesional skin, diffused proteolytic activity was also detected with slightly increased intensity (Figure 3.5 e-h), but not as prominent as that observed in the lesional skin. These results revealed enhanced proteolytic activity in the AD skin. Although we cannot exclude substrate processing by other proteases, these results could still partially represent increased activity of KLK5 in AD. As the initiator of proteolytic cascade in the epidermis, uncontrolled KLK5 activity might also promote the amplification of protease activation cascade and consequently contribute to the elevated proteolytic activity in AD. Furthermore, the activity of epidermal proteases was extended to deeper layer of the AD skin, where diffused expression of KLK5 was also observed, suggesting the abberent proteolytic activity in AD could be attibuted to dysregulation of KLK5.

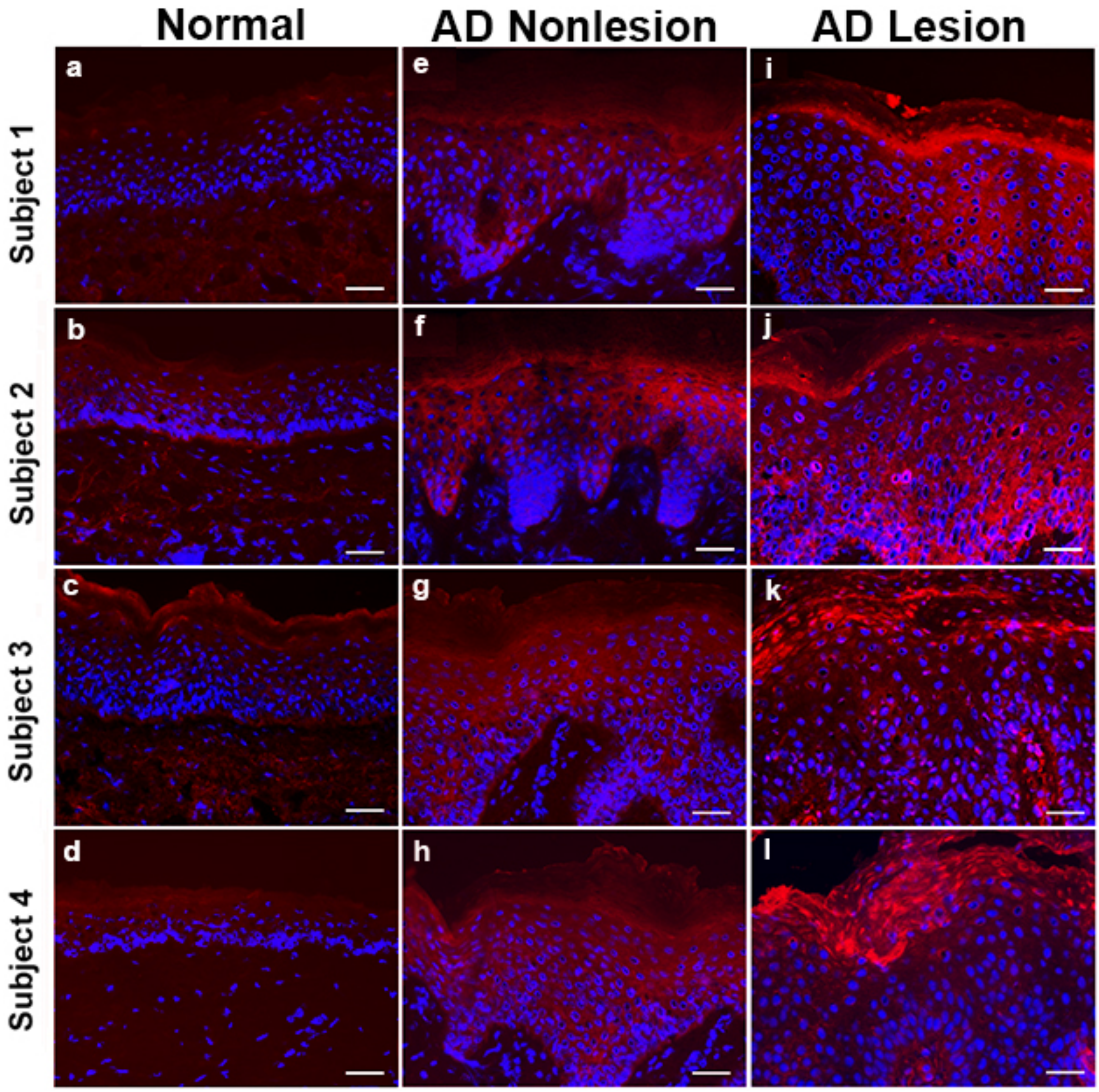

Figure 3.5. Unregulated activity of epidermal proteases in the AD skin.
The activity of total epidermal proteases in the normal and AD skin was examined by in situ zymography. Red color represents total epidermal proteolytic activity. Blue color represents DAPI-stained nuclei._ The proteolytic activity was extended to the deeper layers in the AD lesional skin with increased intensity (i-l) compared to the normal skin (a-d). There was slightly increased protease activity in the AD nonlesional skin (e-h). Bar= 50 µm.

3.1.4 Over-degradation of DSG1 in the AD skin

As KLK5 can cause the proteolytic cleavage of corneodesmosomal protein DSG1, the expression of DSG1 was examined in the AD skin. The antibody that recognizes the extracellular domain of DSG1 was used.

DSG1 was detected in the suprabasal layers of the normal skin with a strong membranous pattern of staining. In contrast, there was decreased staining intensity of DSG1 in the AD lesional skin compared to the normal skin, especially in the granular layer, where the activity of KLK5 might be up-regulated (Figure 3.6 A, a-d vs. i-l). The staining pattern of DSG1 in the nonlesional skin was similar to that in the normal skin, but reduced intensity of DSG1 staining was already detected in the granular layer of nonlesional skin from two subjects (Figure 3.6 A, f and g).

The intensity of DSG1 staining was further quantified. The results showed significant decrease of DSG1 staining intensity in the lesional skin compared to the normal skin ($p<0.05$) (Figure 3.6 B). In contrast, the level of DSG1 was not significantly reduced in the nonlesional skin ($p>0.05$).

It has been reported that as a secreted protease, KLK5 is able to degrade the extracellular adhesion domain of DSG1 through proteolytic cleavage and further induce the epidermal desquamation (Caubet et al. 2004; Descargues et al. 2006). Up-regulation of KLK5 can lead to over-degradation of DSG1 and further result in premature desquamation (Descargus et al. 2006). Combined with these previous findings, reduced level of DSG1 in AD skin indicated the over-degradation of DSG1 potentially caused by the progressive cleavage by up-regulated KLK5.

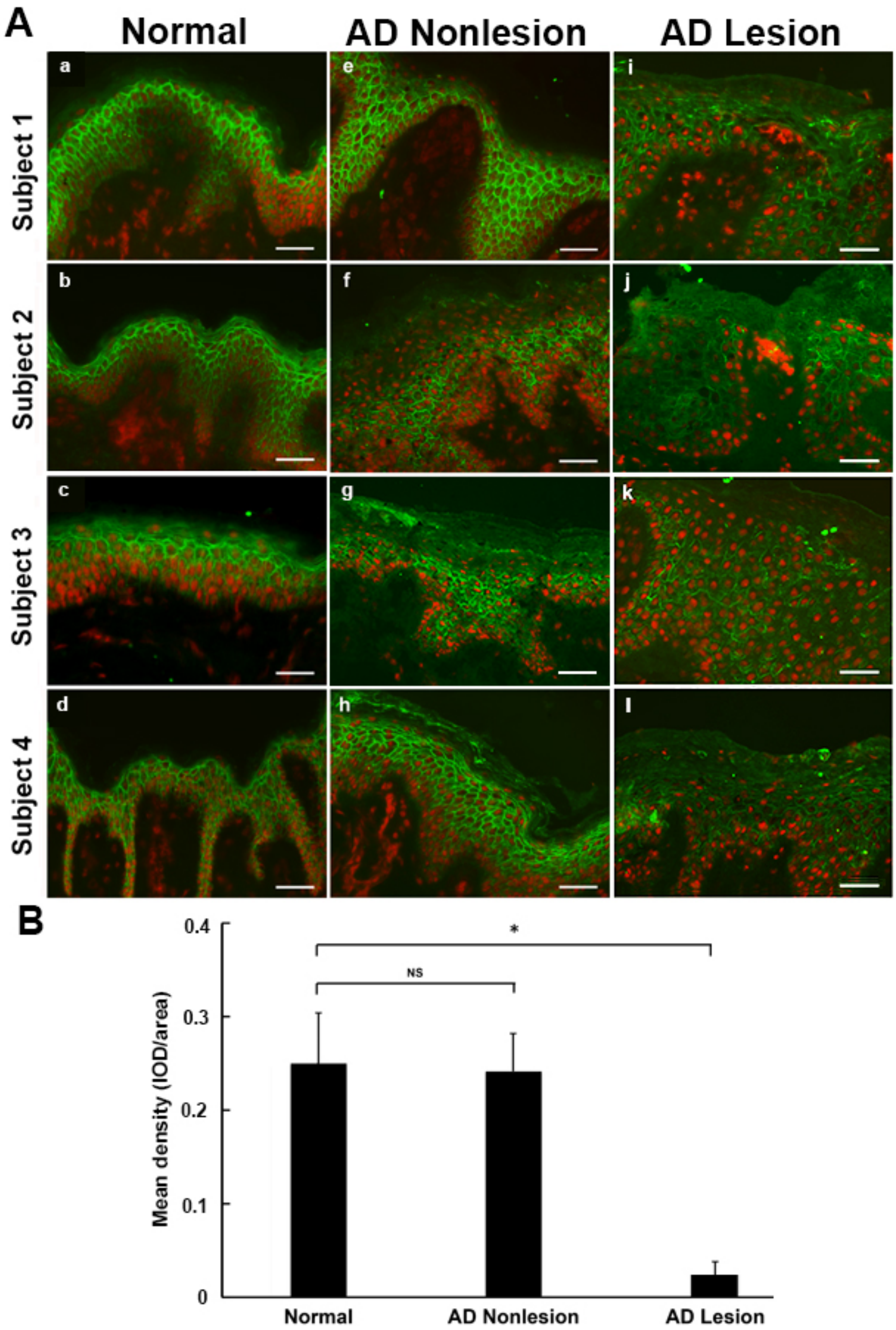

Figure 3.6. Over-degradation of DSG1 in the AD skin.
*Immunofluorescence staining of DSG1 was carried out in the normal and AD skin. Green color represents the staining of DSG1. Red color represents PI-stained nuclei. The intensity of DSG1 staining was reduced in the lesional skin (i-l) compared to the normal skin (a-d). Slightly decreased intensity of DSG1 staining was also observed in the nonlesional skin of two subjects (f,g). Bar= 100 μm (A). Quantification of DSG1 expression in the normal and AD skin was performed (B). Intensity of DSG1 staining was reduced in the lesional skin (n=4) compared to the normal skin (n=4), but there was no significant decrease in the nonlesional skin (n=4). Error bars represent the standard error. * indicates results significant at p<0.05.*

3.1.5 Expression of PAR2 in normal and AD skin

Since KLK5 is an endogenous activator of PAR2, the expression of PAR2 was evaluated in the AD skin. The results showed that PAR2 was localised in the upper granular layer of the epidermis in normal skin. Some non-specific staining was observed within the cornified layer, which was also seen in the negative controls. In comparison, the expression of PAR2 was extended to inner layers of epidermis in the AD skin (Figure 3.7A).

The expression of PAR2 was quantified according to the intensity of PAR2 staining. There were no significant differences in PAR2 expression among normal, AD nonlesional and lesional skin ($p>0.05$) (Figure 3.7B). As the antibody cannot distinguish the active form from the non-active form of PAR2, the results from immunostaining only reflected the total protein level of PAR2 but not the PAR2 function. Therefore, it still remained unclear whether the function of PAR2 is affected by dysregulated KLK5 in AD, which required further investigation.

3.1.6 Reduced level of FLG in the AD skin

Previous study has revealed that up-regulated KLK5 activity is able to trigger the hyperactivity of ELA2, an epidermal serine protease which directly proteolyse both profilaggrin and filaggrin monomers. This results in the deficiency of FLG and consequenly lead to the skin barrier impairment (Bonnart et al. 2010). Therefore, the expression of FLG was also examined in the AD skin, however, the patients recruited in our study have not been genotyped for FLG mutations.

As shown by immunostaining, FLG was expressed in the granular and lower cornified layer in the normal skin. In contrast, the intensity of FLG staining was decreased in the AD lesional skin compared to the normal skin. In addition, the level of FLG was also reduced in the nonlesional skin, but not as prominent as the reduction in lesional skin (Figure 3.8). The intensity of FLG staining was further quantified. The results revealed that the level of FLG was significantly decreased in the lesional skin compared to the normal skin ($p<0.05$), and the intensity of FLG staining was also reduced in the nonlesional skin ($p<0.05$) (Figure 3.9).

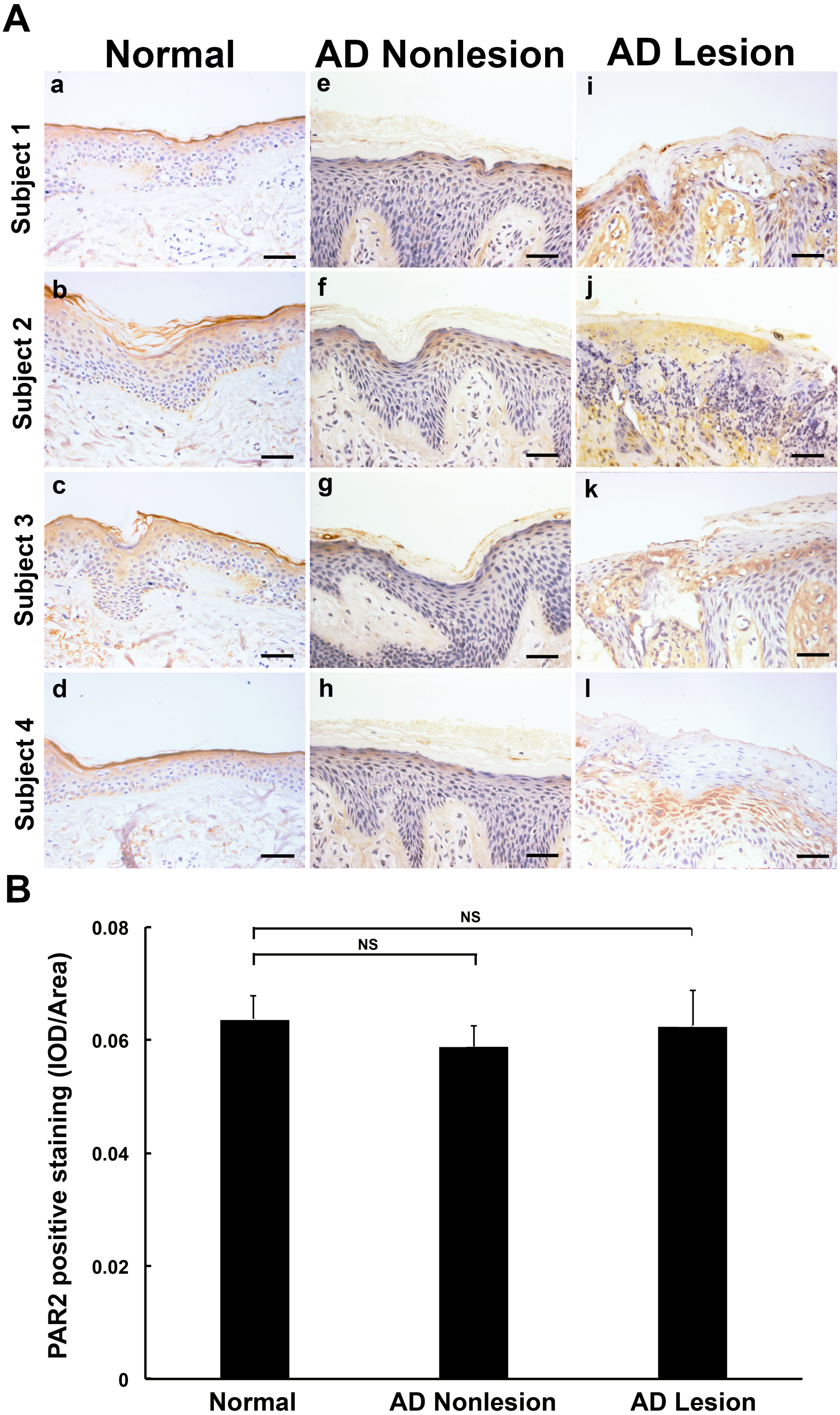

Figure 3.7. The expression of PAR2 in normal and AD skin.
Immunohistochemistry staining of PAR2 was carried out in the normal and AD skin. Brown color represents the staining of PAR2. Blue color represents hematoxylin-stained nuclei. PAR2 expression was extended to the deeper layers of the epidermis in the lesional skin (i-l) compared to the normal skin (a-d). Bar= 50 μm (A). Quantification of PAR2 expression in the epidermis of normal and AD skin was performed (B). The results showed that there was no significant difference of PAR2 expression among the normal skin (n=4), AD nonlesional (n=4) and lesional skin (n=4). Error bars represent the standard error.

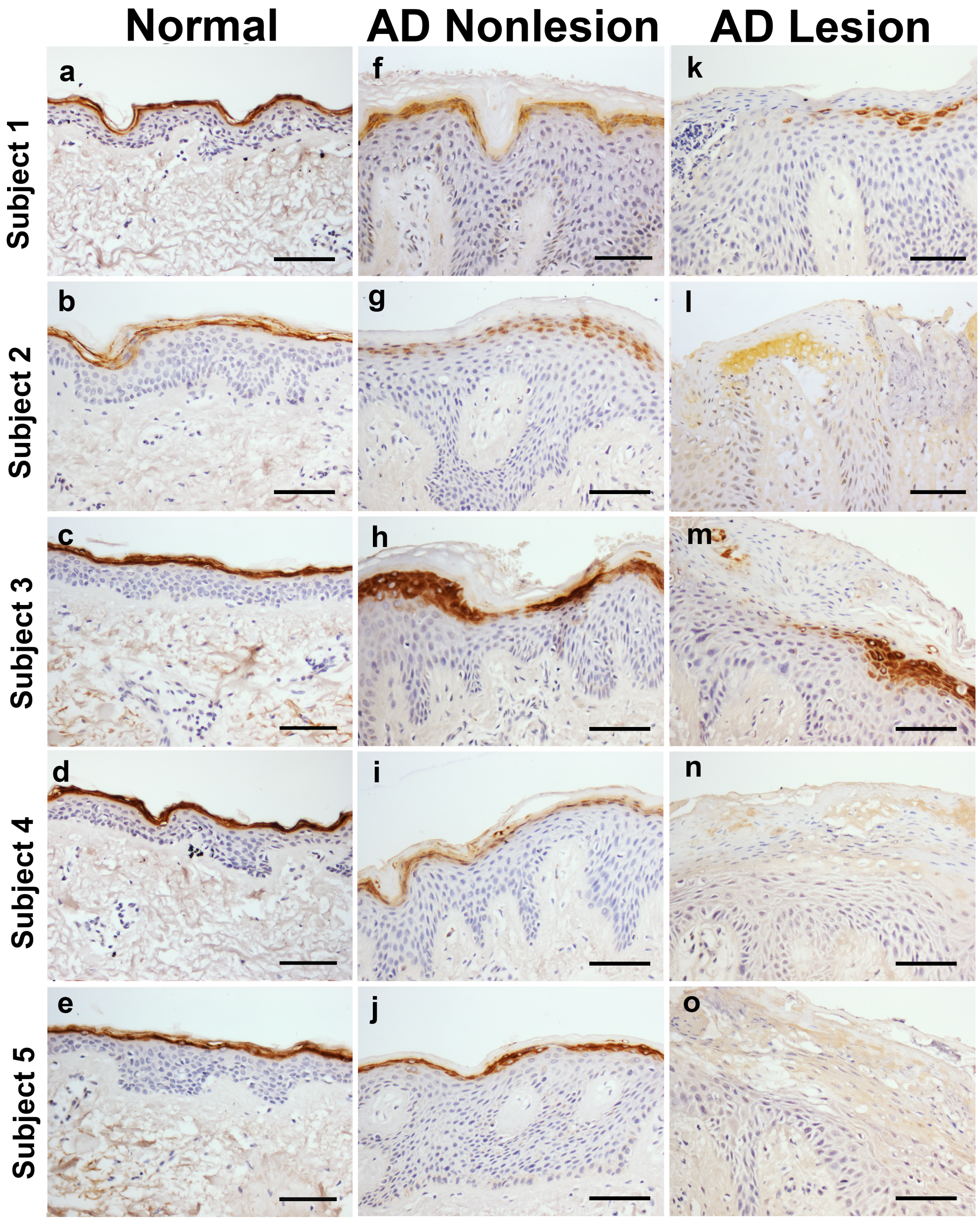

Figure 3.8. Reduced level of FLG in the AD skin.

Immunohistochemistry staining of FLG was carried out in the normal and AD skin. Brown color represents the staining of FLG. Blue color represents hematoxylin-stained nuclei. The expression of FLG was decreased in the lesional skin (k-o) compared to the normal skin (a-e), there was also reduced FLG expression in the nonlesional skin (f-j), but not as prominent as that in the lesional skin. Bar= 50 µm.

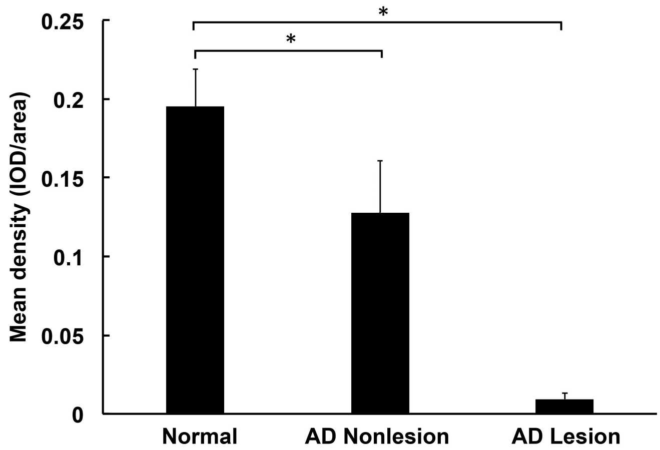

Figure 3.9. Quantification of FLG expression in normal and AD skin.
*The expression of FLG was quantified according to the intensity of FLG staining in the skin biopsies. The results indicated decreased expression of FLG in the lesional skin (n=5) compared to the normal skin (n=5), and there was reduced level of FLG in the nonlesional skin (n=5) as well. Error bars represent the standard error. * indicates results significant at p<0.05.*

3.1.7 Summary

The AD lesional skin exhibited disturbed epidermal morphology, which was characterised by acanthosis, parakeratosis and enlarged intercellular space. Acanthosis is also known as diffused epidermal hyperplasia and characterised by thickened spinous and granular layers, indicating an increased cell number or proliferation of keratinocytes. Parakeratosis is usually seen in diseases of increased cell turnover and is associated with disturbed terminal differentiation. Thus, these histological features implicated disrupted proliferation/differentiation of keratinocytes in AD lesions. Moreover, enlarged intercellular space in the lesional skin could be attributed to lack of adhesion between adjacent keratinocytes or intercellular edema in the epidermis (spongiosis) in AD. Furthermore, reduced levels of barrier-related proteins DSG1 and FLG were also detected in the lesional skin. In addition, the results suggested increased expression and enhanced activity of KLK5 in the AD lesional skin, indicating up-regulated KLK5 might be correlated with the barrier disruption in AD. In the nonlesional skin, impairments of epidermal morphology and dysregulations of KLK5, DSG1, FLG were already observed, but far less prominent than those observed in the lesional skin.

As a secreted protease, KLK5 cleaves extracellular adhesion domain of DSG1 to trigger the epidermal desquamation. In this study, reduced intensity of DSG1 staining was found in the AD skin especially in the granular layer, where the activity of KLK5 might be up-regulated. These results suggested that unopposed KLK5 activity could result in over-degradation of DSG1 and subsequently contribute to deficiency of intercellular adhesion in AD.

Recent studies by other research groups showed that single stimulation by recombinant KLK5 (rKLK5) triggered the activation of PAR2 (Stefansson et al. 2008; Briot et al. 2009). However, exposure to PAR2 stimuli at a supramaximal concentration or repeated stimulation could cause the desensitization and impaired function of PAR2 (Dery et al. 1998; Oikonomopoulou et al. 2006). As a chronic skin disorder, AD is characterized by persistent epidermal barrier defect, which may induce consistent up-regulation of KLK5 (Section 1.4.4). In addtion, the results suggested that increased expression and enhanced activity of KLK5 already existed in the nonlesional skin of some AD pateints. These observations indicated that up-regulation of KLK5 could be sustained in the AD skin, thus the function of PAR2 might be affected by continuous stimulation of consistently up-regulated KLK5 in AD. Currently, there is no *in situ* approach available to examine PAR2 function in the skin, thus an *in vitro* cell model overexpressing KLK5 would be useful to further investigate the influences of sustained KLK5 up-regulation on the function of PAR2.

3.2 Generation and characterization of human keratinocytes overexpressing KLK5

Further experiments were conducted to investigate the contribution of sustained KLK5 up-regulation to the skin barrier impairment in AD. Human keratinocytes ectopically overexpressing KLK5 were generated using lentiviral expression vector. This cell model could mimic the consistent up-regulation of KLK5 in AD and eliminate other causative factors of epidermal barrier defect. KLK5 gene was cloned into the vector containing spleen focus-forming virus (SFFV) promoter and internal ribosome entry site (IRES) linked eGFP reporter gene (KLK5/eGFP). The vector expressing eGFP alone (eGFP) was used as control (Section 2.3). Human keratinocyte cell lines NIKS, NTERT, and primary human keratinocytes were transduced with eGFP vector (eGFP-cells) or KLK5/eGFP vector (KLK5-cells), and untransduced cells (UT) were used as negative control (Section 2.4). The influences of overexpressed KLK5 on its downstream molecules such as DSG1 and PAR2 were evaluated.

3.2.1 Keratinocytes ectopically overexpressing KLK5

Following the exposure to one round of lentiviral infection at multiplicity of infection (MOI) 10, transduction efficiencies in keratinocytes were assessed on the basis of eGFP expression using both fluorescent microscope and flow cytometry. Expression of eGFP was visualised under fluorescent microscope, and eGFP positive cells were found in all three types of keratinocytes transduced with eGFP or KLK5/eGFP vector (Figure 3.10). The transduction efficiencies were 75% for eGFP-cells and 67% for KLK5-cells in NIKS keratinocytes, 72% for eGFP-cells and 69% for KLK5-cells in NTERT keratinocytes, and 64% for eGFP-cells and 62% for KLK5-cells in primary keratinocytes. These results revealed that high transduction efficiencies were achieved in all types of keratinocytes using lentiviral vectors.

The transgene expression in transduced keratinocytes was evaluated on the basis of eGFP expression during the propagation of cells in a period of up to 21 days. The proportions of eGFP positive cells were examined using flow cytometry at indicated time points (Figure 3.11). There was no significantly reduced percentage of eGFP positive cells in both NIKS and NTERT cells transduced with eGFP or KLK5/eGFP vector. As primary keratinocytes cannot be cultured for a long period, the expression of eGFP was only assessed for 12 days. Similarly, the proportion of eGFP positive cells was not significantly decreased in primary cells transduced with eGFP or KLK5/eGFP vector. These results revealed stable transgene expression in keratinocytes mediated by lentiviral transduction. Transduced keratinocytes with over 50% eGFP positive cells were used for further experiments.

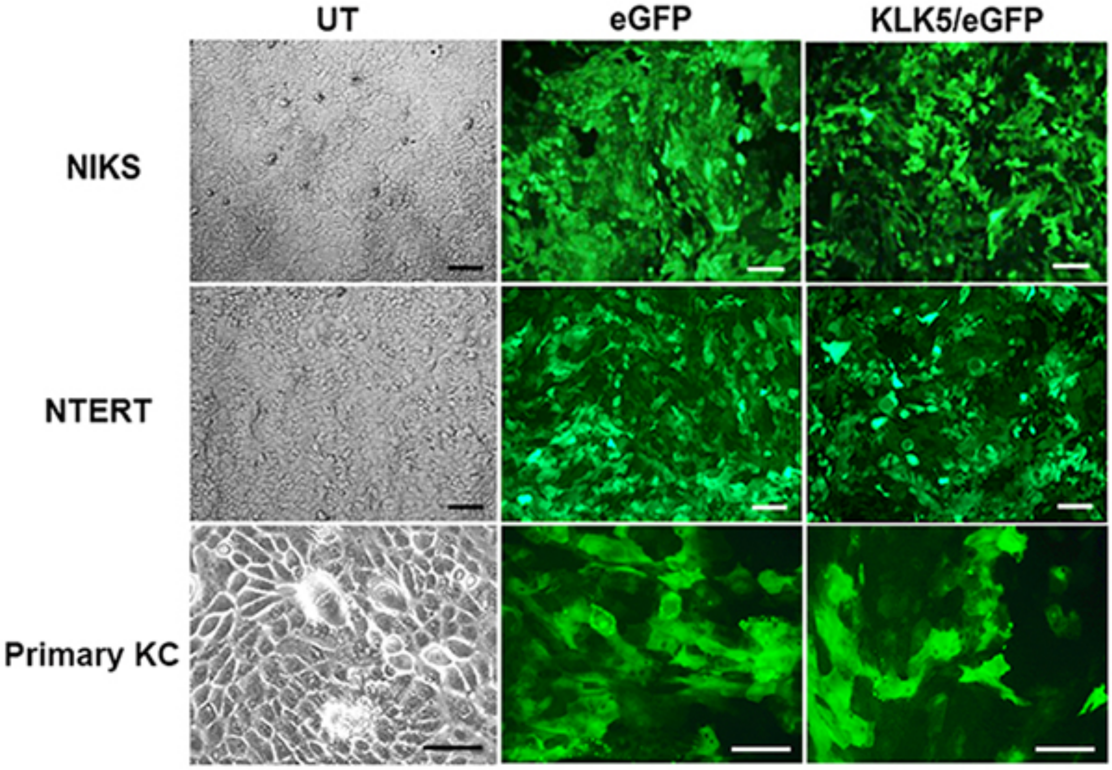

Figure 3.10. Transduction efficiencies in keratinocytes transduced with lentiviral vectors.
The expression of eGFP in transduced NIKS, NTERT and primary human keratinocytes (primary KC) were evaluated using fluorescent microscope and flow cytometry on Day 6 post-transduction. Green color represents GFP fluorescence. eGFP positive cells with green colour were found in all three types of keratinocytes transduced with eGFP or KLK5/eGFP vector. UT: untransduced cells. Bar=100 µm (A).

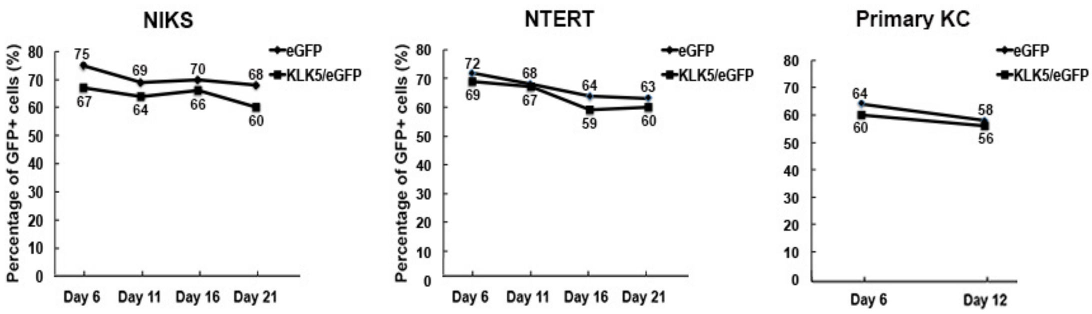

Figure 3.11. The expression of eGFP in transduced keratinocytes.
Percentages of eGFP positive cells in transduced NIKS, NTERT and primary keratinocytes were assessed for a period of up to 21 days using flow cytometry. Stable expression of eGFP was detected in three types of keratinocytes transduced eGFP or KLK5/eGFP vector.

3.2.2 Characterization of KLK5-cells

The expression of KLK5 in transduced NTERT cells was examined using immunocytostaining (Section 2.5). The intensity of KLK5 staining was increased in KLK5-cells compared to untransduced cells and eGFP-cells, suggesting ectopically overexpressed KLK5 in human keratinocytes transduced with KLK5/eGFP vector (Figure 3.12). The results also showed co-localisation of KLK5 and eGFP in cells transduced with KLK5/eGFP vector, whereas no co-expression of KLK5 and eGFP was observed in eGFP-cells.

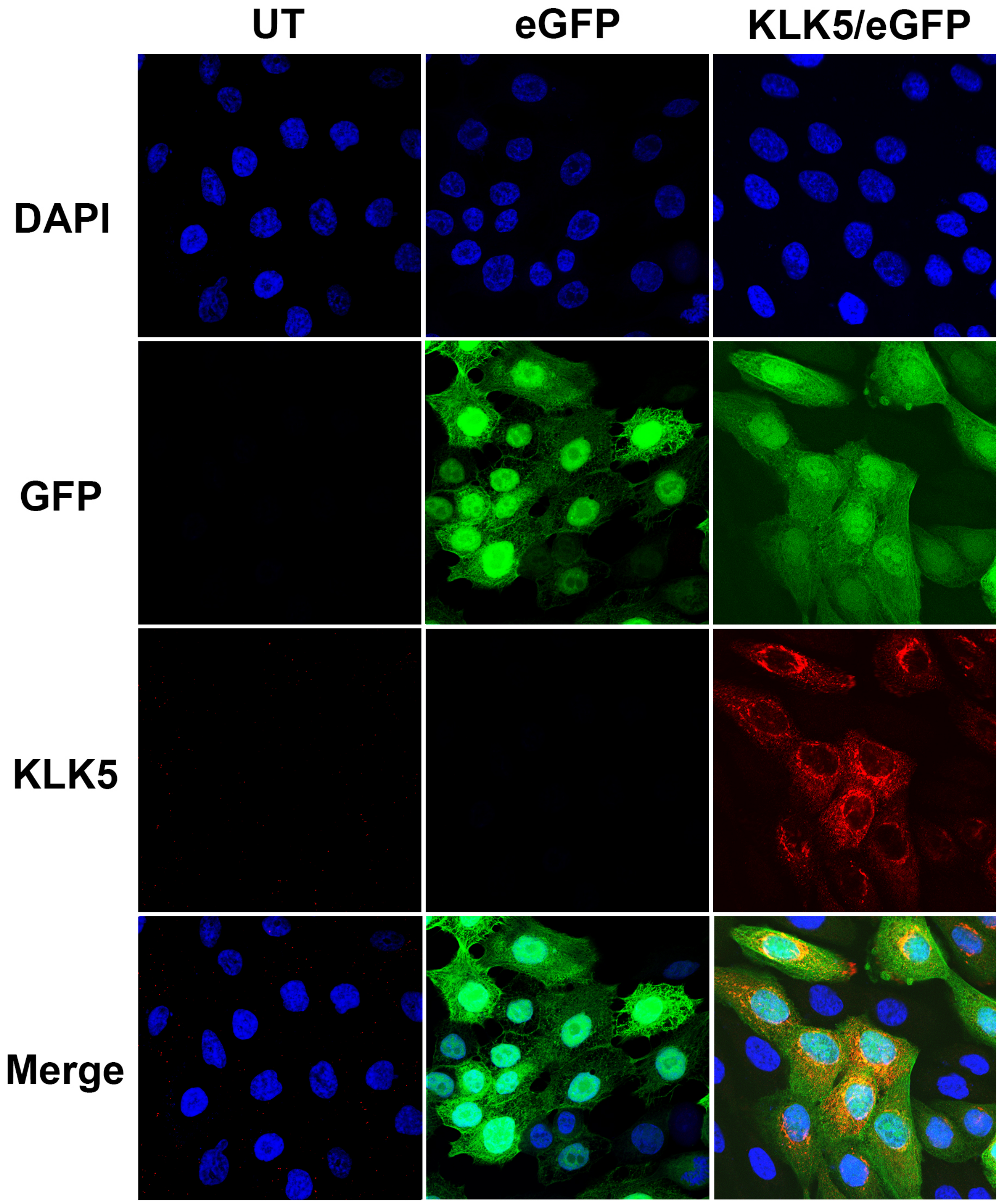

Figure 3.12. Overexpression of KLK5 in keratinocytes transduced with KLK5/eGFP.
The expression of KLK5 in transduced NTERT cells was examined using immunocytostaining. Red color represents the staining of KLK5. Green color represents GFP fluorescence. Blue color represents DAPI-stained nuclei. Increased KLK5 expression was detected in KLK5-cells (KLK5/eGFP) compared to untransduced cells (UT) and eGFP-cells (eGFP). Bar=10 µm.

The levels of KLK5 in transduced NIKS, NTERT and primary keratinocytes were also evaluated by western blotting (Section 2.6). A band at ~38kDa was detected in the cell lysates obtained from untransduced cells, eGFP-cells and KLK5-cells with increased level in KLK5-cells. Protein levels were quantified by densitometry and normalized to β-actin. The results showed significantly increased expression of KLK5 in cells transduced with KLK5/eGFP vector ($p<0.05$) (Figure 3.13).

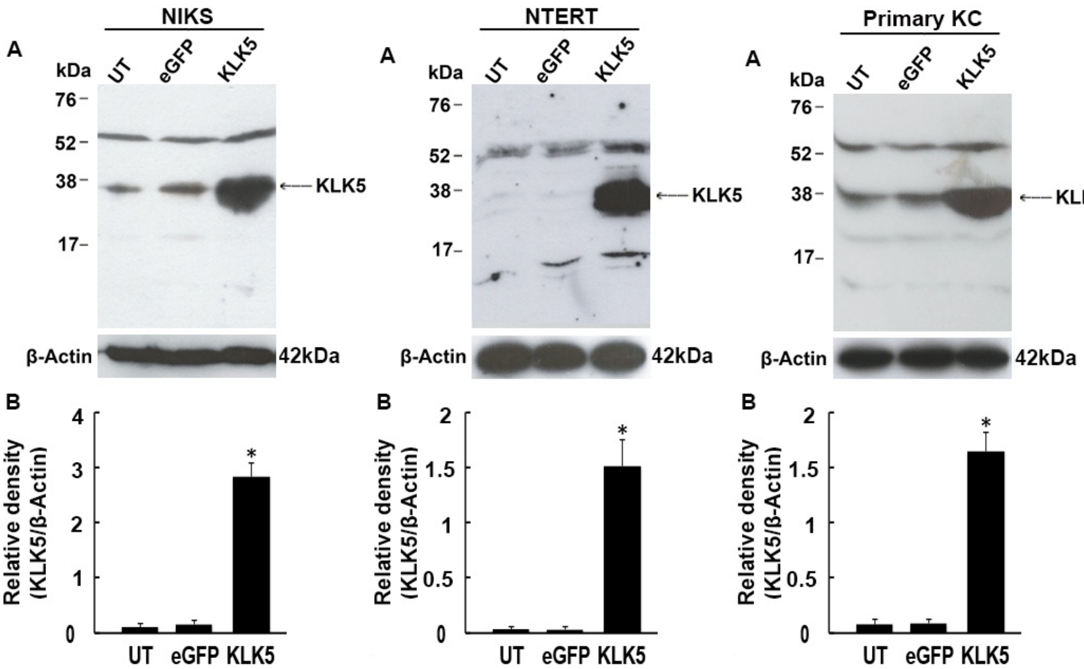

Figure 3.13. Increased expression of KLK5 in the lysates of KLK5-cells.

*The expression of KLK5 in transduced NIKS, NTERT and primary keratinocytes was examined using western blotting. A band at ~38kDa was detected in all the cells with increased level in KLK5-cells. β-Actin was used as loading control. Representative western blot out of three independent experiments (n=3) are shown (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart. There was significantly increased expression of KLK5 in the lysates of keratinocytes transduced with KLK5/eGFP vector compared to untransduced cells (UT) and eGFP cells (B). Error bars represent the standard error. * indicates results significant at p<0.05.*

As KLK5 is a secreted protein, the expression of KLK5 in the culture media collected from NIKS, NTERT and primary keratinocytes was also examined using western blotting. A band at ~34kDa was detected in the culture media collected from untransduced cells, eGFP-cells and KLK5-cells with elevated intensity in KLK5-cells. Protein levels were quantified by densitometry and normalized by Ponceau red staining. The expression of KLK5 was also significantly increased in the culture media of KLK5-cells compared to untransduced cells and eGFP-cells ($p<0.05$) (Figure 3.14). The results of immunocytostaining and western blotting both revealed ectopically overexpressed KLK5 in human keratinocytes transduced with KLK5/eGFP vector.

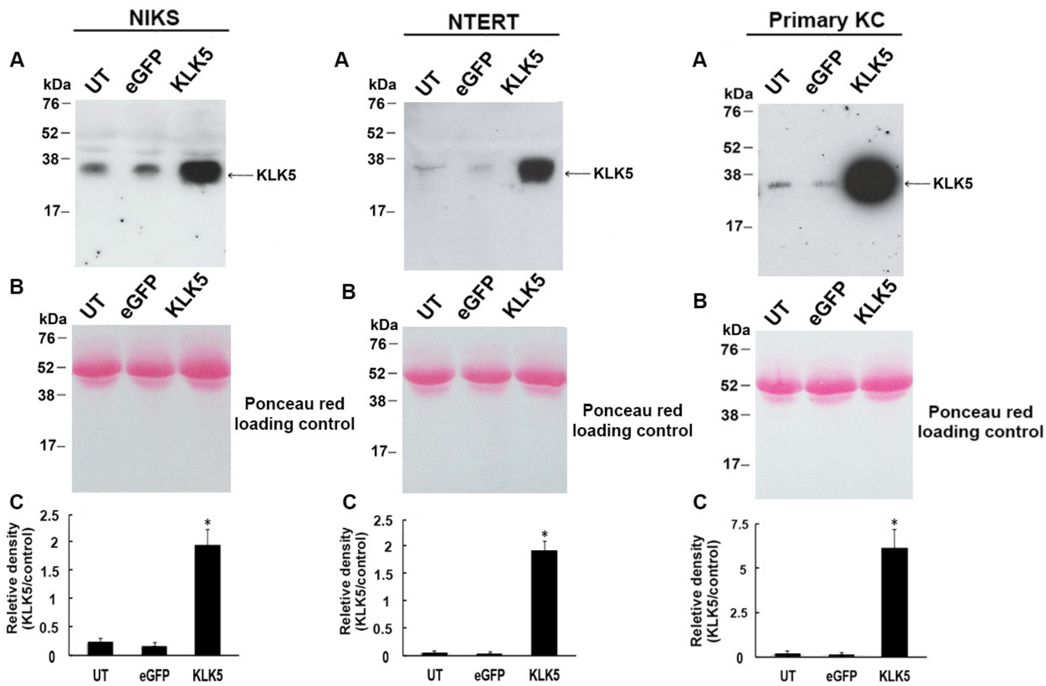

Figure 3.14. Ectopically overexpressed KLK5 in the culture media of KLK5-cells.
*The expression of KLK5 in the culture media obtained from NIKS, NTERT and primary keratinocytes was examined using western blotting. A band at ~34kDa was detected in all the cells with increased level in KLK5-cells (A). Ponceau red staining was used as loading control (B). One representative experiment out of three (n=3) is shown. Similar results were obtained from three independent experiments. Protein levels were quantified by densitometry as shown in corresponding bar chart. The expression of KLK5 was significantly increased in the culture media of KLK5-cells compared to untransduced cells (UT) and eGFP-cells (C). Error bars represent the standard error. * indicates results significant at p<0.05.*

Furthermore, the sizes of KLK5 protein in the cell lysates and culture media were compared. KLK5 showed bands at ~38kDa and ~34kDa in the cell lysates and culture media respectively (Figure 3.15). These two bands might correspond to pro-KLK5 in the cell lysates and active form of KLK5 in the culture media.

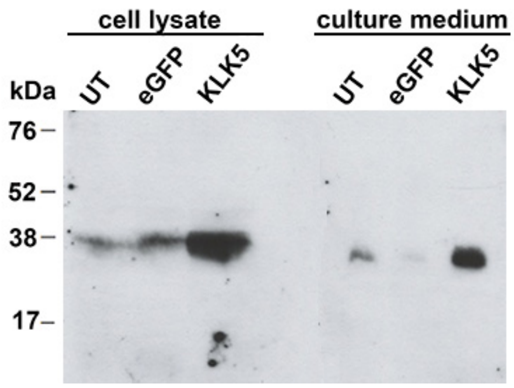

Figure 3.15. The sizes of KLK5 protein in the cell lysates and culture media.
The sizes of KLK5 protein in the cell lysates and culture media collected from NIKS keratinocytes were compared using western blot. The band of KLK5 protein predominantly detected in the culture media was ~4kDa less than that in the cell lysates.

In order to further confirm there was active form of KLK5 in the culture media, the activity of KLK5 in both culture media and cell lysates obtained from NIKS and NTERT keratinocytes was evaluated by casein zymography (Section 2.7). Commercial rKLK5 (active form) was used as positive control.

A digestion band was detected in the culture media collected from KLK5-cells at ~34kDa, which is the same size as the digestion band of commercial rKLK5 (Figure 3.16A, b and d). Meanwhile, same samples were examined by western blotting (Figure 3.16A, a and c). The results confirmed that the size of digestion bands in zymography was consistent with the size of KLK5 bands in western blotting (Figure 3.16A). In contrast, no KLK5 activity was detected in the cell lysates of keratinocytes transduced with KLK5/eGFP vector (Figure 3.16B). The results showed that secreted KLK5 in the culture media of KLK5-cells exhibited proteolytic activity, indicating it was the active form of KLK5.

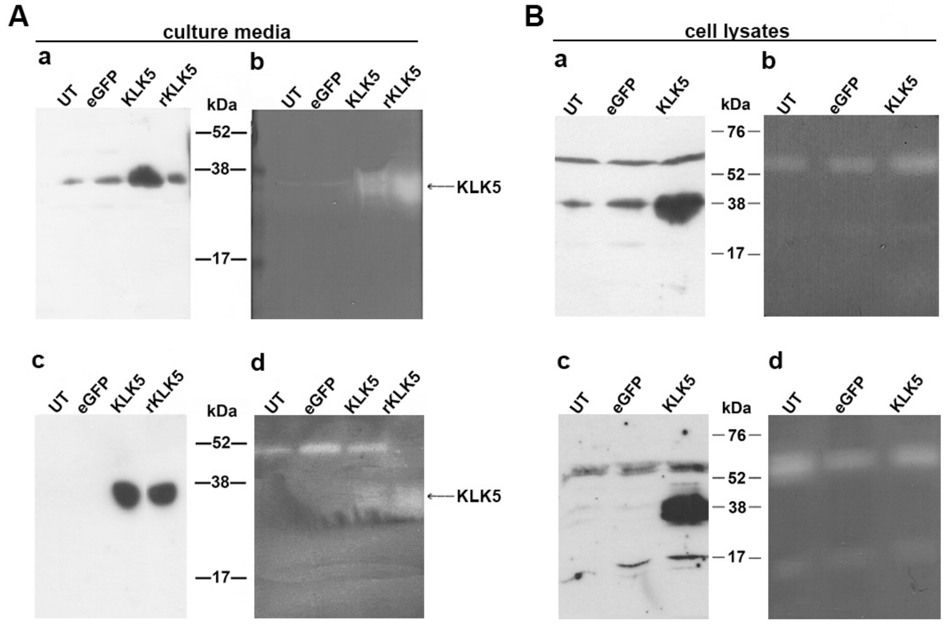

Figure 3.16 Active KLK5 in the culture media of KLK5-cells.
The activities of KLK5 in both culture media and cell lysates were examined using casein zymography and the same samples were also examined by western blotting. Secreted KLK5 in the culture media of KLK5-cells showed bands at the same size as commercial rKLK5 (active form) in both western blotting and zymography (a,b for NIKS cells and c,d for NTERT cells) (A). In comparison, no caseinolytic activity of KLK5 was detected in the cell lysates (B).

3.2.3 The influences of up-regulated KLK5 on DSG1 and PAR2

As both DSG1 and PAR2 are predominantly expressed in differentiated keratinocytes, primary keratinocytes, which possess better potential to undergo proliferation and differentiation *in vitro*, were used to investigate the influences of up-regulated KLK5 on DSG1 and PAR2.

The expression of DSG1 in primary keratinocytes transduced with KLK5/eGFP was examined by western blotting. An antibody recognizing extracellular domain of DSG1 was used. The results showed a specific band of full-length DSG1 at ~165kDa in all the cells with reduced level in KLK5-cells. Protein levels were quantified by densitometry and normalized to β-Actin. There was decreased intensity of DSG1 band in KLK5-cells compared to untransduced cells and eGFP-cells (Figure 3.17), suggesting the over-degradation of DSG1 in keratinocytes overexpressing KLK5.

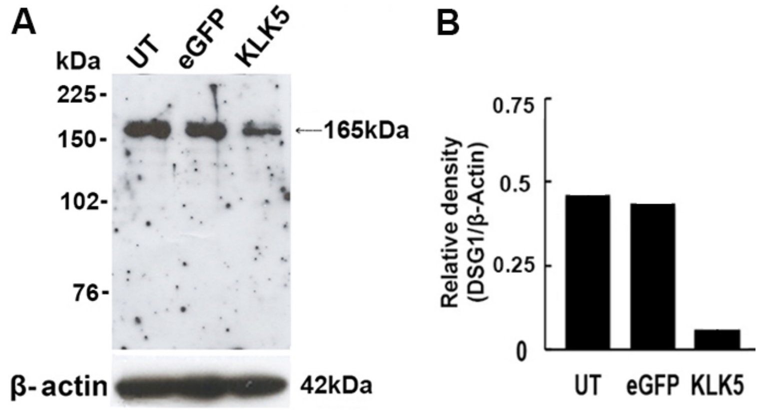

Figure 3.17. Reduced level of DSG1 in KLK5-cells.
The expression of DSG1 in transduced primary keratinocytes was examined using western blotting. A specific band of full-length DSG1 was detected at ~165kDa with reduced intensity in KLK5-cells. β-actin was used as loading control. Representative western blot out of two independent experiments are shown (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart. There was decreased intensity of DSG1 band in KLK5-cells compared to untransduced cells (UT) and eGFP-cells (B).

The expression of PAR2 was examined using anti-PAR2 antibody. Two major bands at ~71kDa and ~55kDa were detected in western blotting. These bands were reported previously, due to considerable N-linked glycosylation of PAR2 on Asn[63] and Asn[555] (Koo et al. 2002; Compton et al. 2009; Aman et al. 2010). Protein levels were quantified by densitometry and the expression of PAR2 was evaluated as a sum of these two bands (71 + 55 kDa). The results showed that PAR2 expression was not changed in KLK5-cells compared to untransduced cells and eGFP-cells (Figure 3.18).

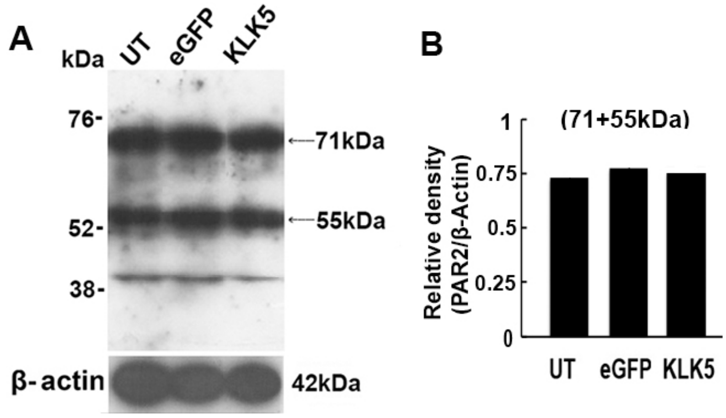

Figure 3.18. Unchanged expression of PAR2 in KLK5-cells.
The expression of PAR2 in keratinocytes was examined using western blotting. Two bands at 71 and 55kDa were detected, corresponding to different glycosylated forms of PAR2. β-Actin was used as loading control. One representative western blot out of two is shown (A). Similar results were obtained from two independent experiments. Protein levels were quantified by densitometry as shown in the corresponding bar chart. PAR2 expression remained unchanged in KLK5-cells compared to untransduced cells (UT) and eGFP-cells (B).

Although the expression of PAR2 was unchanged in KLK5-cells, the influences of consistently overexpressed KLK5 on PAR2 function remained unknown and were further investigated. It has been reported that activated PAR2 can trigger intracellular calcium mobilization and further exert its regulatory function in the cell (Dery et al. 1998; Gardell et al. 2008). Therefore, the function of PAR2 in KLK5-cells was evaluated by measuring the level of PAR2-dependent intracellular calcium mobilization using calcium mobilization assay (Section 2.8).

PAR2 can be activated by its specific agonist peptide (PAR2-AP, SLIGKV-NH_2). In order to validate whether this assay can be used to detect PAR2-dependent intracellular calcium mobilization, normal keratinocytes were stimulated with 100μM PAR2-AP and calcium mobilization was measured in time course. The results showed that intracellular calcium mobilization was initiated immediately following by the injection of 100μM PAR2-AP. Maximal response appeared at ~50 seconds post-injection and calcium mobilization returned to the baseline ~240 seconds post-injection (Figure 3.19). Therefore, PAR2-dependent calcium mobilization could be measured using this assay to reflect the function of PAR2 in keratinocytes.

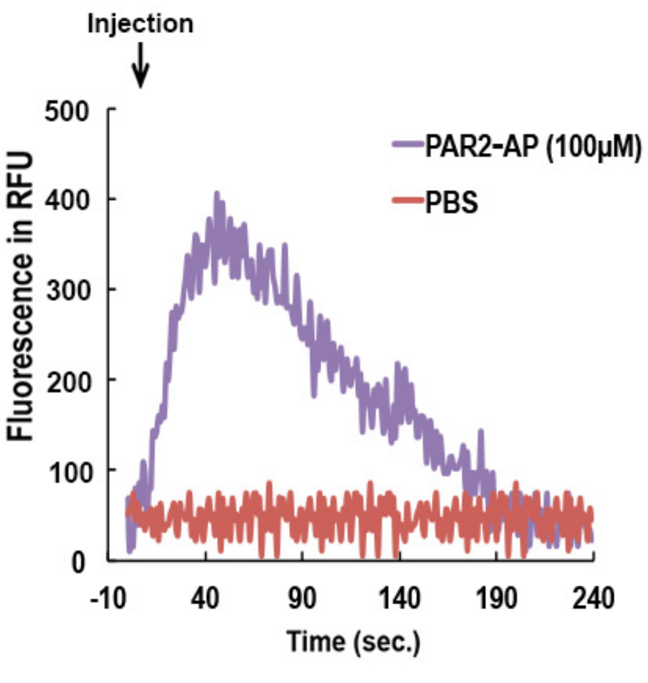

Figure 3.19. Intracellular calcium mobilization in normal keratinocytes followed by the injection of PAR2-AP.
Normal human keratinocytes were injected with 100μM PAR2-AP, and the intracellular calcium mobilization was examined. There was mobilization of intracellular calcium in keratinocytes stimulated with PAR2-AP. Cells injected with PBS only were used as negative control. Similar results were obtained from two independent experiments.

In addition, since single stimulation of KLK5 can also trigger the activation of PAR2, normal keratinocytes were challenged with rKLK5 and calcium mobilization was measured in time course. The results showed that 25μM rKLK5 could stimulate the intracellular calcium mobilization in normal keratinocytes potentially through the activation of PAR2. The mobilization of intracellular calcium was initiated immediately following by the injection of rKLK5. Maximal response appeared at ~110 seconds post-injection and calcium mobilization returned to the baseline ~240 seconds post-injection (Figure 3.20). It was noticed that the response of calcium mobilization was relatively slower in normal keratinocytes challenged with rKLK5 compared to those challenged with PAR2-AP. This could be attributed to the different activating mechanisms of PAR2 mediated by PAR2-AP and KLK5.

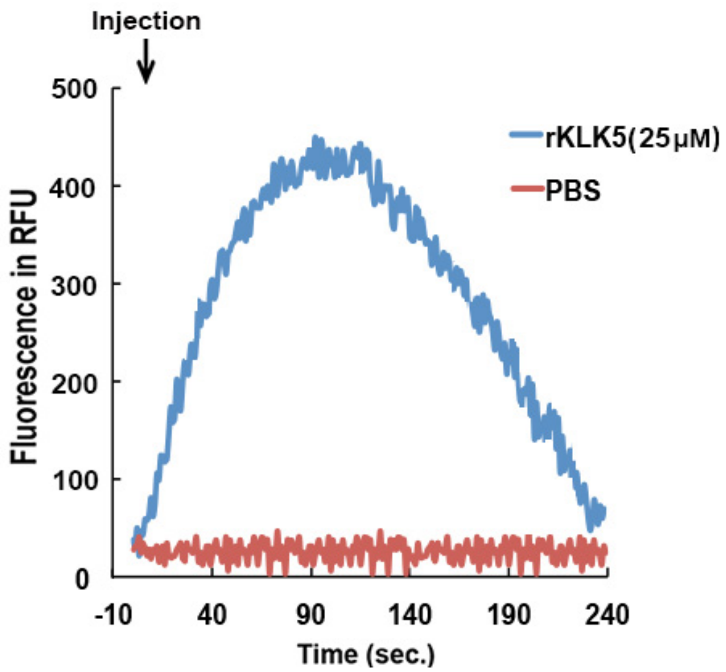

Figure 3.20. Intracellular calcium mobilization in normal keratinocytes stimulated by rKLK5.
Normal human keratinocytes were injected with rKLK5, and the intracellular calcium mobilization was examined. There was mobilization of intracellular calcium in keratinocytes challenged with 25μM rKLK5. Cells injected with PBS only were used as negative control. Similar results were obtained from two independent experiments.

Although single stimulation of KLK5 caused activation of PAR2 in normal keratinocytes, the effects of consistently overexpressed KLK5 on the function of PAR2 still needed to be investigated. Therefore, PAR2-dependent intracellular calcium mobilization was evaluated in KLK5-cells followed by the stimulation of PAR2-AP. Untransduced cells and eGFP-cells were used as controls. PAR2-dependent calcium mobilization was immediately initiated in untransduced cells and eGFP-cells stimulated with PAR2-AP, and the shape of the response curve was similar. However, the level of PAR2-dependent intracellular calcium mobilization was reduced in KLK5-cells compared to the controls (Figure 3.21), indicating the function of PAR2 could be impaired under the repeated stimulation of endogenously overexpressed KLK5.

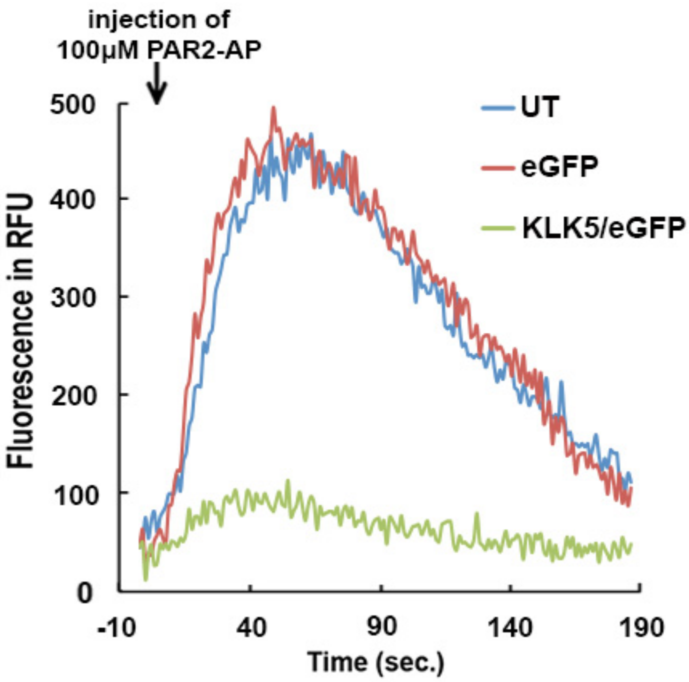

Figure 3.21. Reduced level of PAR2-dependent intracellular calcium mobilization in KLK5-cells. *PAR2-AP was used to challenge untransduced cells, eGFP-cells and KLK5-cells, and the mobilization of intracellular calcium was examined. Decreased PAR2-dependent intracellular calcium mobilization was found in KLK5-cells compared to untransduced cells (UT) and eGFP-cells. Similar results were obtained from two independent experiments.*

Thus, our results showed that single stimulation by exogenous PAR2-AP or rKLK5 could trigger the mobilization of intracellular calcium in normal keratinocytes through the activation of PAR2. However, the level of PAR2-dependent intracellular calcium mobilization was decreased in KLK5-cells, where endogenous KLK5 was consistently overexpressed, suggesting sustained up-regulation of KLK5 might result in impaired PAR2 function in keratinocytes. As exposure to the stimuli at a supramaximal concentration or continuous stimulation could lead to the desensitization of PAR2 (Dery et al. 1998; Oikonomopoulou et al. 2006), it could be theorized that PAR2 was desensitized under the repeated stimulation by overexpressed KLK5, which consequently induced the function impairment of PAR2 in KLK5-cells.

3.2.4 KLK5 overexpression resulted in histopathological features of AD in *in vitro* organotypic cultures

In order to further study the potential influences of overexpressed KLK5 on keratinocytes, organotypic culture (OTC) was used as a skin-equivalent model to evaluate the keratinocyte growth and epidermal formation *in vitro*. OTCs were generated using primary keratinocytes transduced with KLK5/eGFP vector (Section 2.9). Cultures of untransduced cells and eGFP-cells were used as controls. Matured cultures were sectioned to examine the epidermal morphology as well as the protein levels of KLK5, DSG1 and FLG.

The results showed fully developed epidermal structures in the cultures of untransduced cells, eGFP-cells and KLK5-cells (Figure 3.22 a,e,i). eGFP fluorescence could be visualized directly in sections of cultures generated from eGFP-cells and KLK5-cells, particularly within the most superficial cornified layers of the epidermis (Figure 3.22 f,i). eGFP was actually expressed in every layer of the OTCs, however, due to the accumulation of eGFP in the uppermost layers, the eGFP intensity in these layers could result in bleaching effects across other compartments. These results indicated that the SFFV promoter could mediate stable transgene expression in all epidermal compartments with pronounced accumulation of eGFP in the cornified layers.

The culture generated from KLK5-cells exhibited histopathological features of AD by comparing to the normal and eGFP cultures, such as mild parakeratosis and enlarged intercellular space. Furthermore, increased cell sizes of suprabasal keratinocyte was found in the KLK5 cultures (Figure 3.22 a,e,i). KLK5 was localised in the lower cornified layers of the normal and eGFP cultures, whereas the staining of KLK5 was extended throughout the whole epidermis in KLK5 culture. There was increased intensity of KLK5 staining in the culture generated from KLK5-cells, indicating lentiviral-mediated overexpression of KLK5 (Figure 3.22 b,f,j). In addition, DSG1 and FLG were detected in the suprabasal layers of the OTCs of untransduced cells and eGFP-cells. Reduced intensities of DSG1 and FLG staining were found in the cultures generated from KLK5-cells, which were consistent with the decreased levels of these barrier-related proteins in the AD skin (Figure 3.22 c,g,k and d,h,l).

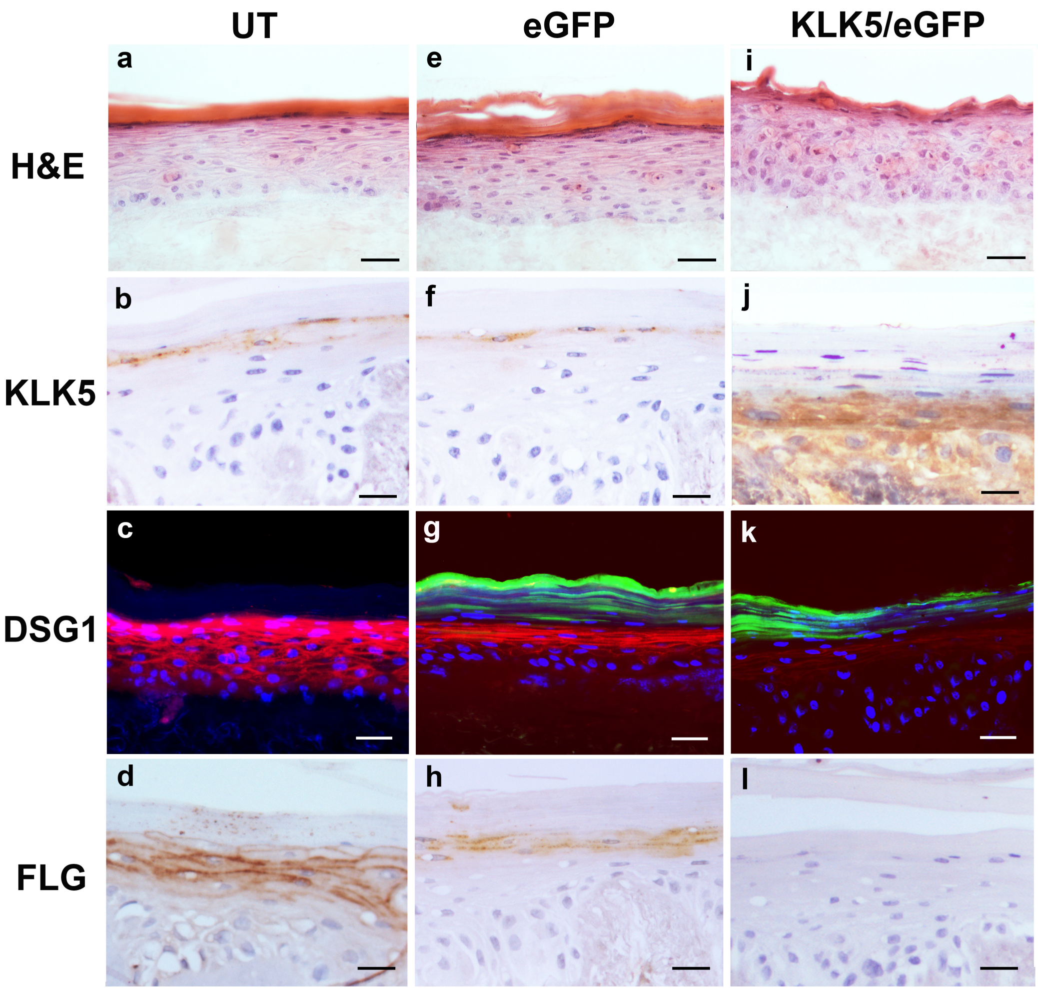

Figure 3.22. Impaired epidermal morphology and disturbed expression of barrier-related proteins in OTCs generated from KLK5-cells.
The skin morphology and the expression of KLK5, DSG1, FLG were examined in the cultures generated from untransduced cells (UT) (a-d), eGFP-cells (eGFP) (e-h) and KLK5-cells (KLK5/eGFP) (i-l) in two independent experiments. b,f,j:Brown color represents the staining of KLK5. Blue color represents hematoxylin-stained nuclei. c,g,k:Red color represents the staining of DSG1. Green color represents GFP fluorescence. Blue color represents DAPI-stained nuclei. d,h,l:Brown color represents the staining of FLG. Blue color represents hematoxylin-stained nuclei. KLK5 cultures exhibited AD-like histopathological features, overexpression of KLK5 and reduced levels of DSG1 and FLG. Bars: (a,e,i and c,g,k) = 100µm; (b,f,j and d,h,l) = 50µm.

3.2.5 KLK5 up-regulation induced AD-like manifestations in *in vivo* skin engraftment model

In order to investigate whether KLK5 overexpression could result in AD-like manifestations, function study of KLK5-cells was also performed using an *in vivo* mouse/human skin engraftment model (Section 2.10). eGFP-cells, KLK5-cells and AD keratinocytes were used to generate the skin grafts. The examination of living mice under fluorescence light revealed eGFP expression as a sign of sustained engraftment (Figure 3.23A, a). At postmortem, no macroscopic abnormalities were detected and histological analysis of the graft and surrounding murine tissue was undertaken (Figure

3.23A, b). Staining for human involucrin allowed areas of human and murine epidermis to be clearly delineated (Figure 3.23B).

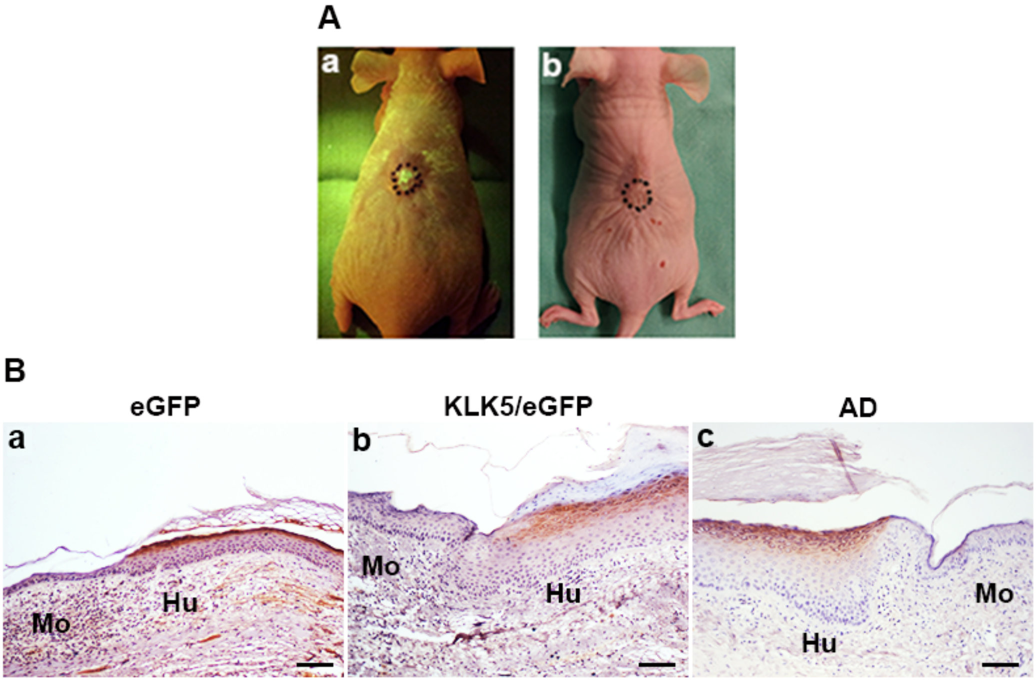

Figure 3.23. In vivo assessment of KLK5-cells using humanized mouse model.
Function study of KLK5-cells was further performed using an in vivo mouse/human skin engraftment model. eGFP-cells, KLK5-cells and AD keratinocytes were used to generate the skin grafts in two independent experiments. A real-time eGFP expression of the graft was evaluated under 488nm light and macroscopic appearance of the graft was examined under transmit light (A). Examination of animals under fluorescence light revealed eGFP expression as a sign of sustained engraftment (a). At postmortem, no macroscopic abnormalities were detected, and histological analysis of the graft and surrounding murine tissue was undertaken (b). Human involucrin expression could indicate mouse (Mo)-human (Hu) skin boundary in all the grafts (B). Brown color represents the staining of involucrin. Blue color represents hematoxylin-stained nuclei. Bar= 100µm.

Histological appearance of the skin grafts was examined using H&E staining. In the skin grafts generated from eGFP-cells, the epidermis exhibited normal architecture (Figure 3.24 a, d). In contrast, the skin grafts generated from KLK5-cells were found to possess histopathological features of AD, including acanthosis, mild parakeratosis and enlarged intercellular space (Figure 3.24 b, e). Similarly, the grafts generated from AD keratinocytes also exhibited similar morphological disruptions as KLK5 grafts (Figure 3.24 c, f). Furthermore, increased cell sizes of suprabasal keratinocytes were also detected in both KLK5 and AD grafts.

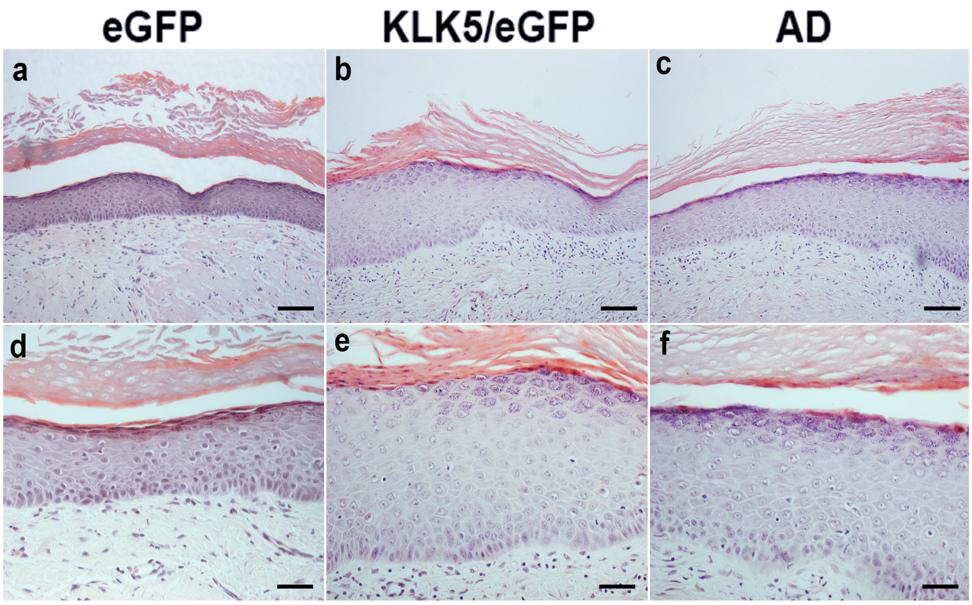

Figure 3.24. AD-like morphological disruptions in the grafts generated from KLK5-cells and AD keratinocytes.
Histological appearance of grafts generated from normal keratinocytes transduced with eGFP (a,d), KLK5/eGFP (b,e) and AD keratinocytes (c,f) in two independent experiments were examined using H&E staining. Both KLK5 and AD grafts exhibited histological features of AD, including acanthosis, parakeratosis and enlarged intercellular space. Bars: a-c=100µm, d-f=50µm.

Furthermore, the expression of KLK5 in the skin grafts was evaluated by immunostaining, and the activity of epidermal proteases was also examined using *in situ* zymography. The results showed that KLK5 was mainly localised in the cornified layer of eGFP grafts, whereas the staining of KLK5 was diffused throughout the whole epidermis with increased intensity in KLK5 grafts. The expression of KLK5 was also elevated and extended to the deeper layers in skin grafts generated from AD keratinocytes (Figure 3.25, a-c). In addition, the results showed that the epidermal proteolytic activity was low and mainly restricted to the cornified layer in eGFP grafts. In comparison, the activity of epidermal proteases was enhanced and extended to the deeper layers in both KLK5 and AD grafts (Figure 3.25, d-f). These results were consistent with the up-regulation of KLK5 and enhanced activity of epidermal proteases observed in the AD skin (Section 3.1.2 and 3.1.3).

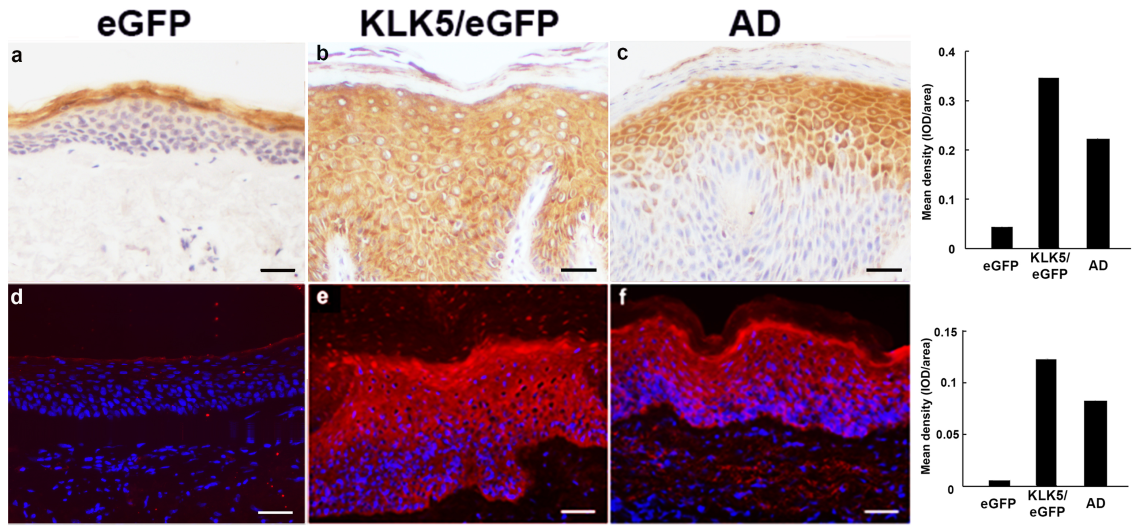

Figure 3.25. Increased expression of KLK5 and enhanced activity of epidermal proteases in the grafts generated from KLK5-cells and AD keratinocytes.
Elevated expression of KLK5 (a-c) and increased activity of epidermal proteases (d-f) were found in both KLK5 and AD grafts compared to eGFP grafts in two independent experiments. One representative experiment out of two is shown. a-c:Brown color represents the staining of KLK5. Blue color represents hematoxylin-stained nuclei. d-f:Red color represents total epidermal proteolytic activity. Blue color represents DAPI-stained nuclei. Bars: a-c=50µm, d-f=100µm.

The expression of DSG1 and FLG was also examined in the skin grafts using immunostaining. DSG1 was detected in the suprabasal layers of eGFP grafts with a strong membranous pattern of staining. The expression of FLG was restricted to the granular and lower cornified layers. There were decreased intensities of DSG1 and FLG staining in both KLK5 and AD grafts compared to eGFP grafts (Figure 3.26). These results were consistent with the reduced levels of DSG1 and FLG in the AD skin (Section 3.1.4), suggesting the skin barrier impairment caused by overexpressed KLK5.

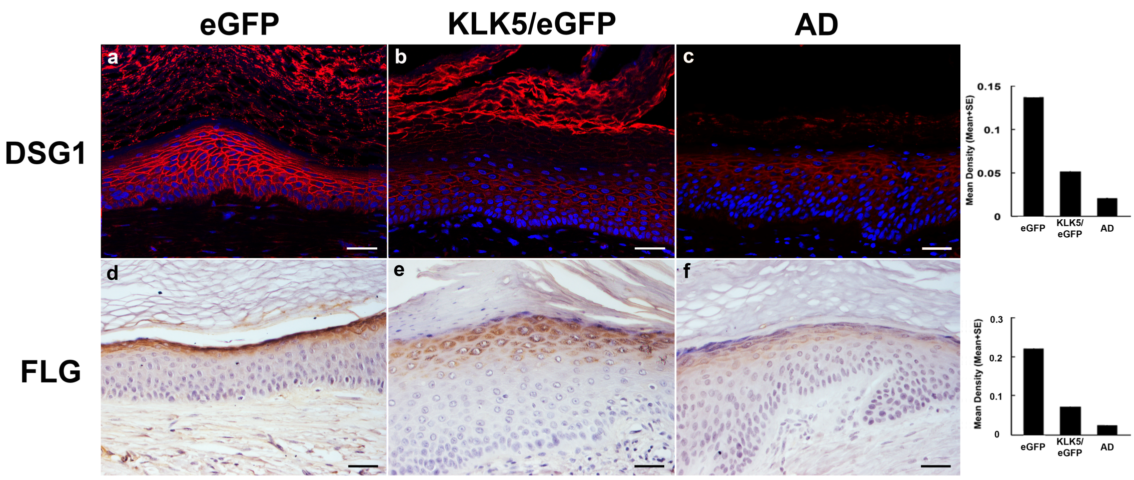

Figure 3.26. Reduced levels of DSG1 and FLG in KLK5 and AD grafts.
Immunostaining for DSG1 (a-c) and FLG (d-f) were performed on the skin grafts generated from two independent experiments. One representative experiment of two is shown. a-c:Red color represents the staining of DSG1. Blue color represents DAPI-stained nuclei. d-f:Brown color represents the staining of FLG. Blue color represents hematoxylin-stained nuclei. Reduced intensities of DSG1 and FLG staining were found in both KLK5 and AD grafts compared to eGFP grafts. Bar = 100 µm.

3.2.6 Summary

Keratinocytes ectopically overexpressing KLK5 were generated using lentiviral vector. High transduction efficiency and stable transgene expression were achieved in transduced keratinocytes. There was increased expression of KLK5 in both cell lysates and culture media collected from KLK5-cells. Casein zymography confirmed that secreted KLK5 in the culture media of KLK5-cells was the active form. Furthermore, the results revealed that consistent up-regulation of KLK5 in keratinocytes resulted in over-degradation of DSG1 and impaired function of PAR2. Both *in vitro* OTCs and *in vivo* skin grafts generated from KLK5-cells exhibited histopathological features of AD including acanthosis, parakeratosis and enlarged intercellular space. In addition, the results showed overexpression of KLK5, enhanced activity of epidermal proteases and reduced levels of DSG1, FLG in the OTCs and skin grafts of KLK5-cells, which were also observed in the AD skin previously (Section 3.1). Therefore, up-regulation of KLK5 in keratinocytes showed the potential to result in AD-like epidermal barrier abnormalities in the skin.

It was noticed that the suprabasal keratinocytes in OTCs and skin grafts generated from KLK5-cells exhibited increased cell sizes compared to eGFP-cells. It has been previously reported that increased sizes of suprabasal cells could reflect disrupted morphological transformation of keratinocytes transitioning into the suprabasal epidermis (Getsios et al. 2009). Combined with these findings, our results indicated that the switch from basal to suprabasal keratinocytes might be disturbed in KLK5-cells. In addition, less flattened cell shape and retention of nuclei in the cornified layer were also found in OTCs and skin grafts generated from KLK5-cells, indicating the impaired keratinocyte differentiation possibly caused by overexpressed KLK5. Abnormal suprabasal morphology and poorly differentiated appearance in OTCs and skin grafts of KLK5-cells suggested that up-regulation of KLK5 might result in disorganised keratinocyte proliferation and differentiation.

Previous studies by other research groups have revealed that single stimulation by KLK5 could activate PAR2 and consequently trigger the overexpression of proinflammatory cytokines (Stefansson et al. 2008; Briot et al. 2009). However, repeated stimulation by endogenously overexpressed KLK5 resulted in impaired function of PAR2 in KLK5-cells. Whether the production of proinflammatory cytokines was affected in KLK5-cells remained unclear and required further investigation.

3.3 Influences of up-regulated KLK5 on keratinocyte growth and cytokine production

It is well established that KLK5 can cause degradation of DSG1 and activation of PAR2 (Caubet et al. 2004; Stefansson et al. 2008). However, results from our *in vitro* and in *vivo* studies using KLK5-cells indicated that consistently up-regulated KLK5 not only induced over-degradation of DSG1, but also showed potential to result in disrupted keratinocyte growth and impaired PAR2 function, which has not been previously reported. As revealed by other studies, single stimulation by recombinant KLK5 could trigger the activation of PAR2 (Stefansson et al. 2008; Briot et al. 2009). Furthermore, activated PAR2 is able to induce the up-regulation of proinflammatory cytokines in keratinocytes (Briot el al. 2009). Actually, up-regulation of KLK5 tends to be consistent in the AD skin. Therefore, single stimulation by KLK5 may not truly reflect the situations in this disease. In contrast, our cell model with sustained overexpression of KLK5, which mimics the situations in AD more closely, could be suitable for studying the roles of up-regulated KLK5 in AD pathogenesis. In order to further investigate the influences of consistent KLK5 up-regulation on keratinocyte growth and cytokine production, expression of relevant genes and proteins in KLK5-cells were examined.

3.3.1 Expression of epidermal growth factor (EGF)-related genes in KLK5-cells

It has been reported that DSG1 is not only a transmembrane adhesion protein serving as a rigid anchor between adjacent cells, but also a potential signalling molecule involved in the regulation of keratinocyte growth (Getsios et al. 2009). DSG1 is required for proper down-regulation of EGF-extracellular signal-regulated kinase 1/2 (Erk1/2) signalling, thereby repressing the proliferation and facilitating the differentiation of keratinocyte. This regulatory function of DSG1 on EGF-pathway relies on its intracellular domain (Getsios et al. 2009). In my previous results, KLK5 from cell lysates showed no proteolytic activity (Section 3.2.2), suggesting the inactive form of KLK5 inside the cell may not interact with the intracellular domain of DSG1. However, KLK5 is activated after being secreted into the extracellular space and active KLK5 is able to degrade the extracellular domain of DSG1. Previous studies have revealed that after the cleavage on extracellular domain, some transmembrane proteins undergo regulated intramembrane proteolysis, which results in the release of intracellular domain fragments from the membrane and further influence the intracellular signalling (Lal & Caplan 2011; Houri et al. 2013). This regulatory event may also occur in DSG1 after the cleavage of its extracellular domain by KLK5. It was hypothesized that over-degradation of DSG1 extracellular domain by up-regulated KLK5 might affect the stability of DSG1 intracellular domain, consequently interfering its regulatory function on EGF-mediated signal transduction. In order to test this hypothesis, the expression of 82 genes related to EGF signalling was examined in keratinocytes overexpressing KLK5 using PCR array (Section 2.11).

Primary human keratinocytes transduced with KLK5/eGFP or eGFP vector with the transduction efficiencies around 60-70% were harvested 6 days post-transduction. The expression of relevant genes was examined in KLK5-cells and compared to that in eGFP-cells. Fold-change values were calculated (Section 2.11). Fold-change values greater than one indicated a positive- or an up-regulation, and fold-change values less than one indicated a negative or down-regulation.

The results showed that 19 genes were up-regulated, whereas 63 genes were down-regulated in KLK5-cells (Appendix 2). Three-fold change was used as the threshold for selection of differential gene expression. The expression of most genes showed no difference between eGFP-cells and KLK5-cells, including EGF and epidermal growth factor receptor (EGFR). The expression of the gene encoding glycogen synthase kinase 3 beta (GSK-3β) was down-regulated by ~6-fold in KLK5-cells compared to eGFP-cells (Figure 3.27). GSK-3β is a protein kinase implicated in the regulation of keratinocyte proliferation and differentiation (Hampton et al. 2012; Chen et al. 2013). It is a regulator in serine/threonine protein kinase Akt pathway, which is involved in EGF-mediated signalling (Galbaugh et al. 2006).

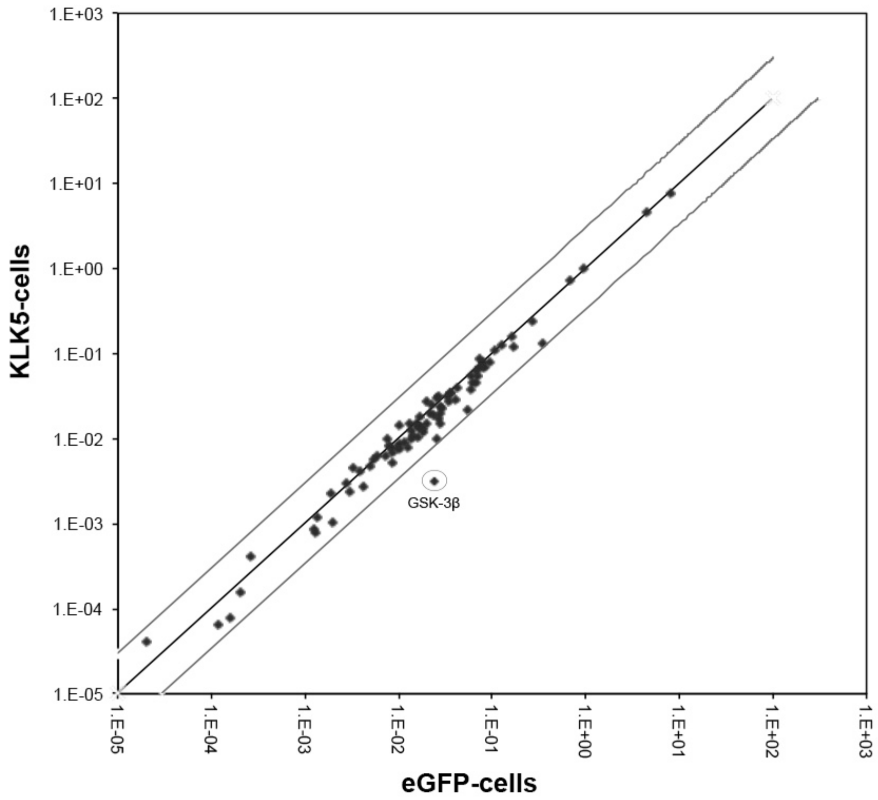

Figure 3.27. Expression of EGF-related genes in KLK5-cells and eGFP-cells.
Human EGF signalling pathway RT^2 profiler PCR array was performed to examine the expression of 82 EGF-related genes in KLK5-cells and eGFP-cells. The scatter plot allowed the visualization of up and down fold-regulation of all genes investigated. The line in the middle indicates fold change of 1 and the other two lines represents fold change of 3. The experiment was performed once. The results showed that the expression of GSK3β was down-regulated by ~6-fold in KLK5-cells compared to eGFP-cells.

3.3.2 Evaluation of GSK-3β expression in KLK5-cells

In order to confirm the findings of PCR array, mRNA level of GSK-3β in KLK5-cells was further examined using RT-PCR (Section 2.12). Untransduced cells and eGFP-cells were used as controls. The results confirmed increased mRNA level of KLK5 in keratinocytes transduced with KLK5/eGFP vector compared to untransduced cells and cells transduced with control vector. However, the mRNA level of GSK-3β remained unchanged in KLK5-cells (Figure 3.28B). The results from RT-PCR were not consistent with the down-regulated mRNA level of GSK-3β in the PCR array.

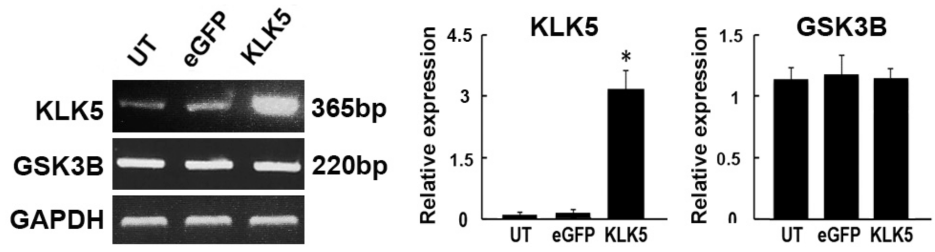

Figure 3.28. mRNA levels of KLK5 and GSK-3β in KLK5-cells.
*mRNA levels of GSK-3β and KLK5 were measured by RT-PCR. The band intensities were quantified by densitometry and normalized to GAPDH. Increased mRNA level of KLK5 was detected in KLK5-cells, whereas the mRNA level of GSK-3β B in KLK5-cells was not changed compared to untransduced cells (UT) and eGFP-cells. One representative experiment out of three (n=3) is shown. Error bars represent the standard error. * indicates results significant at p<0.05.*

The protein level of GSK-3β was also examined using western blotting. There was a band of GSK-3β at ~46kDa in untransduced cells, eGFP-cells and KLK5-cells. Protein levels were quantified by densitometry and normalized to β-actin. The expression of GSK-3β was not changed in KLK5-cells compared to untransduced cells and eGFP-cells (Figure 3.29).

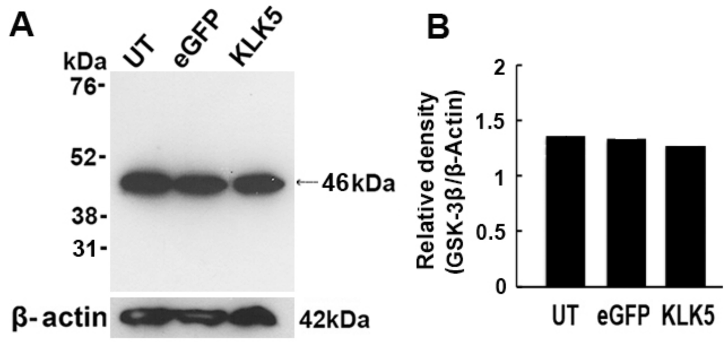

Figure 3.29. Unchanged expression of GSK-3β in KLK5-cells.
The expression of GSK-3β in KLK5 cells was examined using western blotting. A band at ~46kDa was detected in all the cells. Representative western blots out of two independent experiments are shown. Protein levels were quantified by densitometry and normalized to β-Actin. The results showed that the expression of GSK-3β remained unchanged in KLK5-cells compared to untransduced cells (UT) and eGFP-cells.

The results of RT-PCR and western blotting suggested that both mRNA and protein levels of GSK-3β were not changed in KLK5-cells, which were not consistent with the reduced expression of GSK-3β in the PCR array. Thus down-regulation of GSK-3β in KLK5-cells detected by the array may be false positive. The reason could be low reaction efficiency caused by the technical errors or primers degradation, which resulted in decreased level of GSK-3β in the PCR array. Therefore, these results indicated that up-regulated activity of KLK5 in keratinocytes might not affect the expression of GSK-3β.

3.3.3 Expression of protein kinases in KLK5-cells

The growth of keratinocytes is regulated by various signaling pathways, which involve large amount of protein kinases (Calautti et al. 1995, 2005; Dahler et al. 2001; Eckert et al. 2002; Mammucari et al. 2005). Up-regulated KLK5 may affect the process of keratinocyte growth through certain kinases. However, only a limited number of kinase genes related to EGF-mediated signaling were evaluated in the PCR array (Section 3.3.1). Therefore, human phospho–kinase antibody array that contains numerous kinases relevant to keratinocyte growth such as Akt, Fyn, JNK, p38 was also performed (Calautti et al. 1995, 2005; Eckert et al. 2002). The levels of 43 phospho-kinases and 2 related total proteins heat shock protein 60 (HSP60) and β-catenin were examined in KLK5-cells and compared to those in eGFP-cells (Section 2.13).

Primary human keratinocytes were transduced with KLK5/eGFP or eGFP vector. KLK5-cells and eGFP-cells with transduction efficiencies about 50%-60% were harvested 6 days post-transduction. Cell lysates were prepared and subjected to the antibody array. The results showed that 7 proteins were abundantly expressed in both eGFP-cells and KLK5-cells, including p53, CREB, β-catenin, STAT3, WNK1, PRAS40 and HSP60 (Figure 3.30A). The intensities of the blots were quantified using densitometry (Appendix 3). The expression of the target proteins was normalized to the reference protein. Normalized protein levels of phospho-kinases in eGFP-cells and KLK5-cells were compared. The results showed that the expression of phospho-p53 and HSP60 was increased in KLK5-cells compared to eGFP-cells (Figure 3.30B).

The kinases involved in EGF-mediated signalling such as EGFR and Erk1/2 were also included in the antibody array. Binding of EGF triggers the phosphorylation and activation of EGFR and Erk1/2. However, there were no significant differences of phospho-EGFR and phospho-Erk1/2 between KLK5-cells and eGFP-cells, indicating the activities of these EGF-related kinases were not affected by overexpressed KLK5. Together with unchanged expression of EGF and EGFR in KLK5-cells detected by the PCR array (Section 3.1.1), these results suggested EGF-mediated signalling was not influenced by up-regulated KLK5 in keratinocytes.

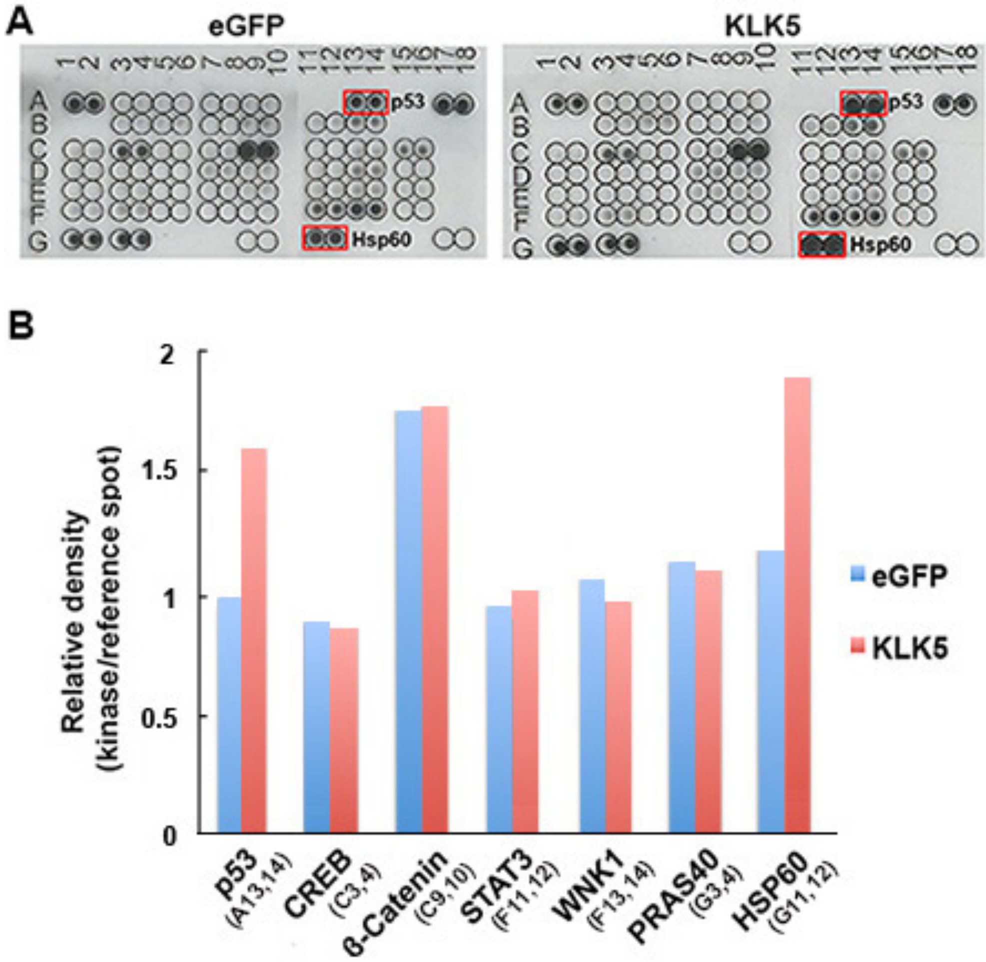

Figure 3.30. The levels of phospho-kinases in KLK5-cells and eGFP-cells.
The expression of 43 phospho-kinases and 2 related total proteins HSP60 and β-catenin in KLK5-cells were examined by comparing to those in eGFP-cells using human phospho-kinase array. Each reference/target was blotted in duplicate. Abundant expression of 7 phospho-kinases was detected in both cells (A). Mean pixel density of each reference/target was calculated. The expression of the target kinases was normalized to the reference protein. Increased expression of p53 and HSP60 was found in KLK5-cells compared to eGFP-cells (B).

3.3.4 Up-regulation of p53 may contribute to disturbed keratinocyte growth in KLK5-cells

The level of phospho-p53 in KLK5-cells was further evaluated using western blotting. The antibody recognizes p53 phosphorylated at S392, same as the one used in the phospho-kinase antibody array (Section 2.17). A band at ~53kDa was detected in untransduced cells, eGFP-cells and KLK5-cells, and the intensity of this band was quantified by densitometry and normalized to β-actin. The results showed increased expression of phospho-p53 in KLK5-cells compared to untransduced cells and eGFP-cells (Figure 3.31). Overexpression of KLK5 in keratinocytes induced elevated level of phospho-p53, which was consistent with the results of human phospho-kinase antibody array.

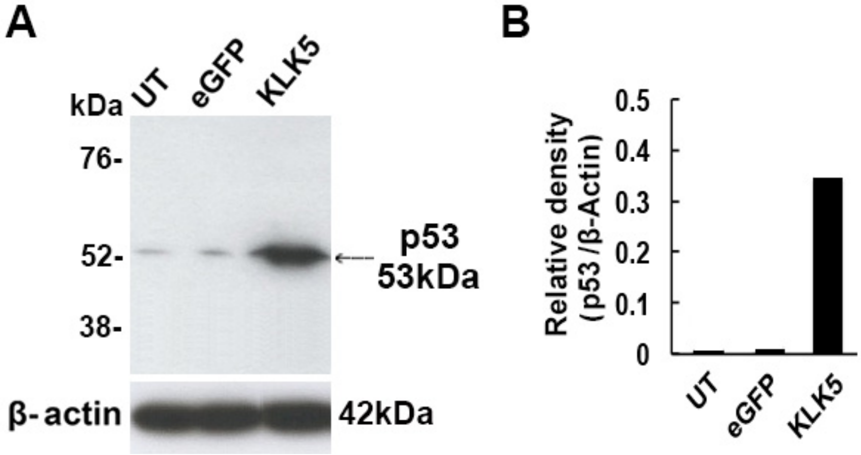

Figure 3.31. Up-regulation of phospho-p53 in KLK5-cells.
The expression of phospho-p53 in KLK5-cells was examined using western blotting. A band at ~53kDa was detected in all the cells with increased level in KLK5-cells. β-Actin was used as loading control. Representative western blot out of two independent experiments are shown (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). Elevated expression of p53 was found in KLK5-cell compared to untransduced cells (UT) and eGFP-cells. Error bars represent the standard error.

p53 is a transcription factor that regulates the cell cycle by targeting numerous growth-related genes, therefore it is implicated in multiple processes such as growth arrest and apoptosis (Piette et al. 1997; Schwartz et al. 1997; Freedman et al. 1999). p53 is also known as tumour protein p53, and the association between p53 and cancer has been well established. In normal cells, the p53 protein is continually produced, but p53 levels are kept low due to continuous degradation by MDM2 (Piette et al. 1997; Freedman et al. 1999). MDM2 is one of the transcriptional targets of p53, which can inactivate p53 protein by forming the complex with it in a negative feedback loop, consequently triggering the degradation of p53 by the proteasome through ubiquitin system and in turn down-regulating p53 (Piette et al. 1997; Freedman et al. 1999). In response to oxidative stress, deregulated oncogene expression and DNA damage induced by UV irradiation or chemical agents, p53 starts to dissociate with MDM2. Then p53 becomes accumulated and activated (Piette et al. 1997; Freedman et al. 1999). Once activated, p53 exerts three major functions: growth arrest, DNA repair and apoptosis. It induces cell growth arrest by holding the cell cycle at the G1/S regulation point to stop the progression of cell cycle, preventing replication of damaged DNA (Piette et al. 1997; Schwartz et al. 1997). During the growth arrest, p53 may activate the transcription of proteins involved in DNA repair. It can also initiate the apoptosis to discard the cells containing damaged DNA (Piette et al. 1997; Schwartz et al. 1997; Freedman et al. 1999). Mutations of p53 have been found in most skin cancers and in more than 50% of all human malignancies, where its role of defencing against cancer is lost (Bieging et al.

2014). The regulatory role of p53 in normal tissue homeostasis has also been revealed. Previous study has reported that maximum expression of p53 was found in proliferating keratinocytes, and down-regulation of p53 was detected in keratinocytes undergoing the transition from proliferation to differentiation (Dazard et al. 2000). These findings suggested that p53 might be abundantly expressed in proliferating keratinocytes, and the level of p53 was down-regulated when cells start to differentiate. Therefore, the level of p53 is delicately maintained during the growth of keratinocytes. Up-regulated level of p53 could be correlated with increased proliferation and disturbed differentiation in keratinocytes overexpressing KLK5.

3.3.5 Elevated HSP60 could induce aberrant cytokine production in KLK5-cells

3.3.5.1 Increased expression of HSP60 in KLK5-cells

The protein level of HSP60 in KLK5-cells was also examined by western blot analysis. The antibody was same as the one used in the phospho-kinase antibody array (Section 2.17). A band at ~60kDa was detected in untransduced cells, eGFP-cells and KLK5-cells with increased level in KLK5-cells. Protein levels were quantified by densitometry, and the expression of HSP60 was increased in KLK5-cells compared to untransduced cells and eGFP-cells (Figure 3.32). These results confirmed the up-regulation of HSP60 in KLK5-cells detected by the antibody array.

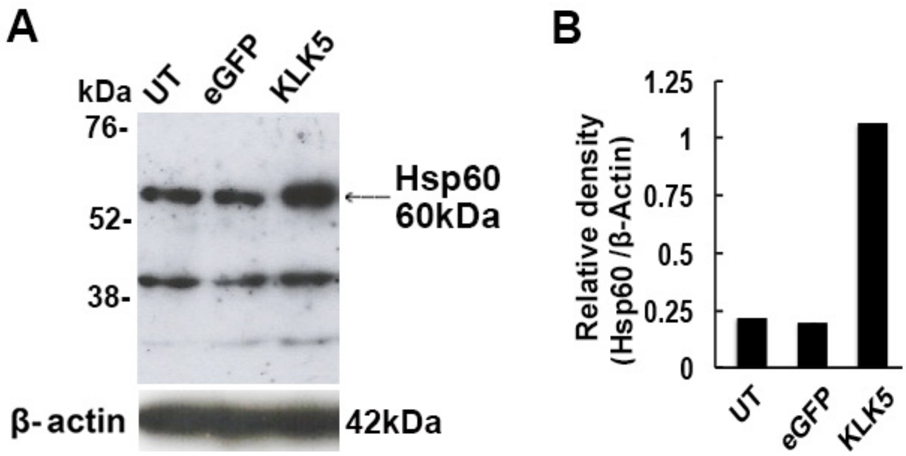

Figure 3.32. Elevated expression of HSP60 in KLK5-cells.
The expression of HSP60 in KLK5-cells was examined using western blotting. A band at ~60kDa was detected in all the cells with elevated level in KLK5-cells. β-Actin was used as loading control. Western blot represented the results of two independent experiments (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). Increased expression of HSP60 was found in KLK5-cells compared to untransduced cells (UT) and eGFP-cells. Error bars represent the standard error.

HSP60 is a mitochondrial chaperonin, which is implicated in the maintenance and transport of mitochondrial proteins (Henderson & Pockley 2010). It also plays a potential role as a "danger signal" in the immune response, which is able to trigger the cytokine secretion and initiate inflammatory response in numerous cell types including macrophages, monocytes and cardiomyocytes (Srivastava 2002; Kang et al. 2013; Tian et al. 2013). Stimulation by exogenous HSP60 can be sensed by toll-like receptor 4 (TLR4), which triggers the activation of various protein kinases such as p38 and MAPK kinase 3, consequently stimulating NFkB-mediated signalling and promoting the production of proinflammatory molecules such as TNFa, IL-12, IL-15, IL-6 and IL-1b (Srivastava 2002; Kang et al. 2013; Tian et al. 2013). Furthermore, in macrophages and dendritic cells, TLR4-mediated signalling is activated and inflammatory cytokines are up-regulated not only in response to exogenous HSP60 but also to endogenous HSP60 produced by stressed cells (Bulut et al. 2002; Quintana & Cohen 2005; Osterloh et al. 2007). It still remained unclear whether HSP60 is involved in the regulation of cytokine secretion in keratinocytes. However, several studies have revealed that TLR4 is expressed in human keratinocytes (Song et al. 2002; Pivarcsi et al. 2003; Lebre et al. 2007). These findings raise the possibility that HSP60 might trigger the production of inflammatory cytokines in keratinocytes through TLR4-mediated signalling. Up-regulation of KLK5 could result in increased level of endogenous HSP60 in keratinocytes and consequently affect the production of cytokines.

3.3.5.2 Elevated production of IL-8, TSLP and IL-10 in KLK5-cells

Both PAR2 and HSP60 are involved in the regulation of cytokine production. KLK5 could affect the secretion of cytokines in keratinocytes through PAR2 or HSP60. In order to investigate the profile of cytokine production in KLK5-cells, the levels of 36 cytokines were evaluated and compared to those in eGFP-cells using human cytokine antibody array (Section 2.14).

Culture media collected from both KLK5-cells and eGFP-cells were subjected to the array. The results showed that expression of 6 cytokines were abundantly detected in the culture media obtained from KLK5-cells and eGFP-cells, including GROa, IL-1ra, IL-8, IL-10, MIF and Serpin E1 (Figure 3.33A). The intensities of the blots were quantified using densitometry (Appendix 4). The expression of the target proteins was normalized to the reference protein. Normalized protein levels of the cytokines in eGFP-cells and KLK5-cells were compared. There was increased expression of IL-8 and IL-10 in KLK5-cells compared to that in eGFP-cells (Figure 3.33B).

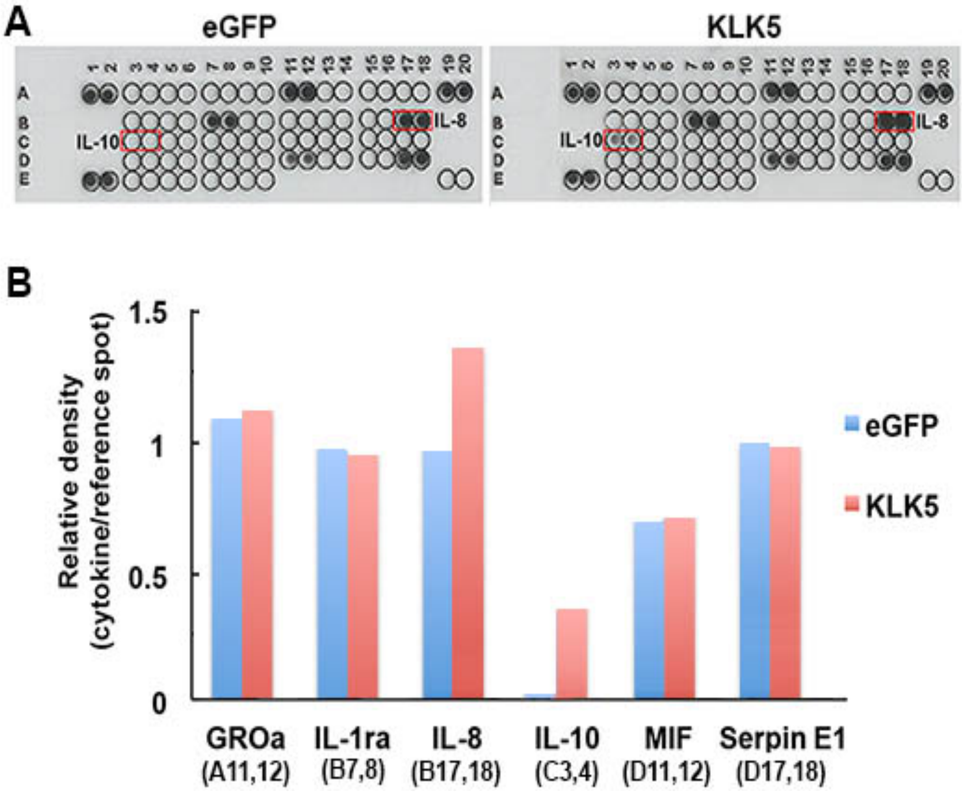

Figure 3.33. Expression of cytokines in KLK5-cells and eGFP-cells.
The expression of cytokines in KLK5-cells was examined using human cytokine array. eGFP-cells were used as control. Each reference/target was blotted in duplicate. Abundant expression of 6 cytokines was detected in both cells (A). Mean pixel density of each reference/target was calculated. The expression of the target cytokines was normalized to the reference protein. Increased expression of IL-8 and IL-10 was found in KLK5-cells compared to eGFP-cells (B).

Expression of IL-8 and IL-10 in KLK5-cells was further examined in samples from three independent experiments using ELISA (Section 2.14). In addition, TSLP is a proinflammatory cytokine involved in the inflammatory response in many skin disorders (Soumelis et al. 2002; Roan et al. 2012). Up-regulation of TSLP is associated with AD-like phenotype, and increased level of TSLP was also found in the lesional skin of AD patients (Yoo et al. 2005; Briot et al. 2009; Sano et al. 2013). These findings suggest TSLP is potential mediator involved in the pathogenesis of AD. As TSLP is not included in the human cytokine antibody array, the level of TSLP in KLK5 cells was evaluated by ELISA as well.

The results showed significantly increased levels of IL-8 and IL-10 in KLK5-cells compared to untransduced cells and eGFP-cells ($p<0.05$) (Figure 3.34 A&C). These results were consistent with the up-regulation of IL-8 and IL-10 in KLK5-cells in human cytokine antibody array. The production of TSLP in KLK5-cells was also significantly elevated compared to that in untransduced cells and eGFP-cells ($p<0.05$) (Figure 3.34 B).

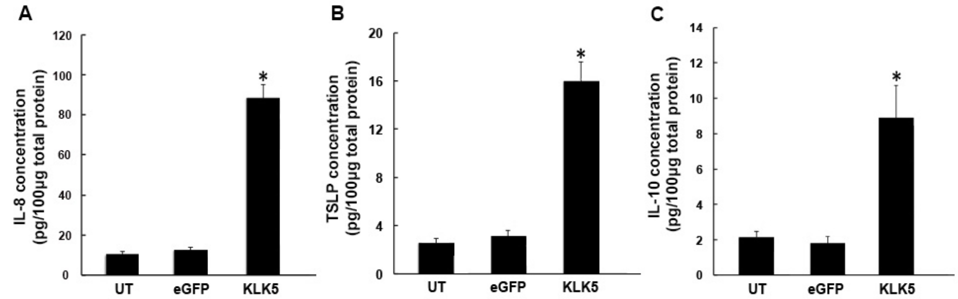

Figure 3.34. Increased levels of IL-8, TSLP and IL-10 in KLK5-cells.
*The expression of IL-8 (A), TSLP (B) and IL-10 (C) in the culture media collected from KLK5-cells was examined using ELISA. Untransduced cells (UT) and eGFP-cells were used as controls. Increased expression of IL-8, TSLP and IL-10 was detected in KLK5-cells compared to untransduced cells and eGFP-cells. Data represent mean±s.e.m. from three independent experiments (n=3). Error bars represent the standard error. * indicates results significant at p<0.05.*

As both PAR2 and HSP60 can regulate the cytokine secretion, dysregulations of PAR2 and HSP60 in KLK5-cells may affect the production of cytokines. It has been reported that KLK5 can trigger overexpression of proinflammatory cytokines such as IL-8 and TSLP in keratinocytes through PAR2-mediated signalling (Briot et al. 2009). However, my previous data revealed that the function of PAR2 was impaired under the repeated stimulation by endogenously overexpressed KLK5 (Section 3.2.3), which could not result in the up-regulation of IL-8 and TSLP in KLK5-cells. Therefore, increased levels of IL-8 and TSLP in KLK5-cells might be attributed to other factors such as up-regulated HSP60.

Keratinocyte-derived IL-8 functions as a chemoattractant to cause the migration of human neutrophils and T cells toward the site of inflammation (Barker et al. 1991). Previous studies implicate the production of IL-8 is regulated by TLR4-NFkB signalling (Pivarcsi et al. 2003; Sawa et al. 2008). It is possible that overexpressed KLK5 causes up-regulation of HSP60 in keratinocytes, which could stimulate TLR4-NFkB-mediated signal transduction, consequently result in elevated level of IL-8 and contributing to the inflammatory infiltrate in skin.

TSLP is a pro-Th2 cytokine. It can initiate the differentiation of Th2 cells and promote the production of Th2 cytokines (Liu et al. 2007; Roan et al. 2012; Jang et al. 2013). Overexpression of TSLP has been found in AD lesions (Sano et al. 2013), which could contribute to the Th2 immune response in this skin disease. It has been reported that TLR4-NFkB pathway potentially involves in the expression of TSLP (Li et al. 2011). It was speculated that overexpression of KLK5 could trigger TLR4-NFkB signalling through up-regulating HSP60, subsequently leading to increased level of TSLP in keratinocytes. This may trigger the Th2 response and contribute to the immune dysregulation. As a result, up-regulated Th2 cytokines possibly leads to reduced level of lipid production and decreased amount of corneodesmosomes in the epidermis (Sawada et al. 2012; Hatano et al. 2013), consequently abrogating the intercellular cohesion and exacerbating the epidermal barrier dysfunction.

IL-10 is a cytokine primarily produced by monocytes and Th2 cells, and expression of IL-10 is also found in keratinocytes. It has been reported that IL-10 exerts its anti-inflammatory function by suppressing the production of Th1 proinflammatory cytokines such as interferon-γ (IFN-γ) (Fiorentino et al. 1991; D'Andrea et al. 1993). Previous study revealed that apart from its inhibition on the Th1 response, IL-10 was also able to promote the Th2 response in allergic skin inflammation (Laouini et al. 2003a). Increased level of IL-10 has been detected in the AD skin (Ohmen et al. 1995; Howell et al. 2005). In immune cells, the level of IL-10 can be regulated by groups of transcription factors including NFkB, STATs, GATA3 and SMAD4 (Saraiva & O'Garra 2010). It is not clear whether the IL-10 production in keratinocytes is regulated through the same pathways. Up-regulated KLK5 in keratinocytes may stimulate the activation of transcription factors such as NFkB through HSP60-mediated signalling, consequently resulting in elevated secretion of IL-10 and promoting Th2 immune response.

Sustained overexpression of KLK5 induced increased production of IL-8, TSLP and IL-10 in keratinocytes possibly through up-regulating HSP60. In order to confirm the speculation, future experiments need to be carried out to elucidate this potential signalling mediated by KLK5 and HSP60.

3.3.6 Summary

The expression/activity of EGF, EGFR and Erk1/2 remained unchanged in KLK5 cells, suggesting DSG1-EGF signalling and its regulatory role in keratinocyte growth was not affected by up-regulated KLK5. However, there was increased level of p53 in keratinocytes overexpressing KLK5. However, p53 up-regulation in KLK5-cells could also be a response to *in vitro* culture. Therefore, whether p53 is up-regulated in a KLK5-dependent manner still needs to be confirmed. As p53 is down-regulated during the switch of keratinocytes from proliferation to differentiation, up-regulation of p53 could be correlated with disrupted cell proliferation and differentiation, consequently contributing to disturbed keratinocyte growth in KLK5-cells. The level of HSP60 was also elevated in keratinocytes with sustained KLK5 overexpression. Since HSP60 is able to promote the production of inflammatory cytokines, up-regulation of HSP60 may contribute to increased secretion of IL-8, TSLP and IL-10 in KLK5-cells.

These results indicated that up-regulation of KLK5 not only induced over-degradation of corneodesmosomes, but also showed potential to result in disorganised keratinocyte growth through p53 and lead to aberrant cytokine production in keratinocytes through HSP60. These abnormalities could lead to disturbed epidermal barrier formation and contribute to the immune dysregulation, consequently exacerbating skin barrier defect. Therefore, inhibition of unregulated KLK5 activity in keratinocytes using potential inhibitor might be an effective strategy for the restoration of epidermal barrier function.

3.4 Inhibition of up-regulated KLK5 reversed the abnormalities in barrier-related proteins

Sustained overexpression of KLK5 induced over-degradation of DSG1 and up-regulation of p53 in keratinocytes, which could consequently result in the impaired epidermal barrier integrity. Consistent up-regulation of KLK5 also caused increased expression of HSP60 and further resulted in aberrant secretion of inflammatory cytokines in keratinocytes, subsequently contributing to the immune dysregulation. The abnormalities in these barrier-related proteins caused by overexpressed KLK5 could exacerbate skin barrier defect and promote the development of AD. Therefore, KLK5 appeared to be a potential target for the therapeutic approaches of AD. It was speculated that inhibition of up-regulated KLK5 might correct the aberrant expression/activity of its downstream molecules, further improving the function of epidermal barrier and reversing the disease phenotype in AD.

SFTI-1 is a cyclic peptide and the smallest natural serine protease inhibitor. It shows exceptionally potent trypsin-inhibitory activity. SFTI-1 and its analogues exhibit inhibition upon a range of serine proteases including cathepsin G7, matriptase and KLKs (Long et al. 2001; Tan el al. 2013). SFTI-1 is considered as a natural template and a promising peptide-based drug bioscaffold that can be modified to produce highly specific inhibitors (Boy et al. 2010; Lesner et al. 2011). For instance, previous studies have reported that the SFTI-1-derived peptides can selectively block the proteolytic activity of KLK4 and KLK7 (Swedberg et al. 2009, 2011; De Veer et al. 2013). These findings raised the possibility that SFTI-1 could be used as a natural scaffold to generate potential KLK5-specific inhibitors.

In this project, we collaborated with Dr. Macmillan's group at UCL Chemistry Department. They demonstrated that the circular peptides could be prepared via a native chemical ligation-type process. This strategy was used for the first time to produce the analogue of SFTI-1, which was designated as SFTI-G. The inhibitory activity of SFTI-G against KLK5 was confirmed by spectrofluorimetric assay. SFTI-G exhibited inhibition towards KLK5-catalyzed proteolysis of fluorogenic substrate (Boc-Val-Pro-Arg-AMC), and IC_{50} was ~15 μM. Further experiments were conducted in this project to inhibit the unopposed activity of endogenously overexpressed KLK5 using SFTI-G and to investigate whether the impairments in KLK5-cells could be reversed once the KLK5 activity is inhibited. Synthesized SFTI-G was purified and freeze-dried at UCL Chemistry Department. Mass spectrometry analyses showed successful synthesis of

SFTI-G analogue (Shariff et al 2014). Lyophilised peptides were reconstituted in ultrapure water to make the stock solutions, which were then sterilized by filtration through 0.22 μm porous Millipore filters. The stock solutions were aliquoted and kept frozen. At the time of treatment, the solutions were diluted in pre-warmed culture medium to the final concentrations used for the experimental treatments.

3.4.1. The activity of KLK5 was inhibited by SFTI-G

3.4.1.1 SFTI-G inhibited the activity of exogenous recombinant KLK5

Previous results in this study suggested that single stimulation by recombinant active KLK5 was able to induce PAR2-dependent intracellular calcium mobilization in normal keratinocytes (Section 3.2.3). In addition, recombinant KLK5 secreted by cells overexpressing KLK5 (mKLK5) was proven to be the active form (Section 3.2.2), thus mKLK5 should also be able to trigger the intracellular calcium mobilization in normal keratinocytes. The serum-free culture media collected from KLK5-cells was concentrated (Section 2.7). This concentrated media containing mKLK5 was able to trigger calcium mobilization in normal keratinocytes (Figure 3.35). If the activity of mKLK5 can be inhibited by SFTI-G, mKLK5-stimulated intracellular calcium mobilization in keratinocytes should be reduced in the presence of SFTI-G. In order to confirm this, concentrated culture media containing mKLK5 was pre-incubated with SFTI-G (concentrations ranged from 10nM to 100 μM) at 37^{o}C for 10 minutes. The intracellular calcium mobilization was measured in normal keratinocytes followed by the injection of pre-incubated mixtures of mKLK5 and SFTI-G. Cells injected with mKLK5 alone were used as control.

The results showed that intracellular calcium mobilization was initiated immediately followed by the injection of mKLK5 alone. SFTI-G did not affect KLK5-induced intracellular calcium mobilization at the concentration of 10nM. Calcium mobilization in keratinocytes induced by KLK5 was suppressed by SFTI-G at or above the concentration of 100nM. As the concentration of SFTI-G continuously increased, the suppression of calcium mobilization was more obvious. KLK5-induced intracellular calcium mobilization in keratinocytes was effectively suppressed by SFTI-G at the concentration of 100 μM.

It could be theorized that SFTI-G inhibited the activity of KLK5, which prevented the proteolytic cleavage and activation of PAR2 by KLK5. Consequently, reduced PAR2 activation resulted in decreased PAR2-dependent intracellular calcium mobilization in normal keratinocytes, which was observed in the presence of SFTI-G. In addition, SFTI-G with higher concentration was able to inhibit the activity of KLK5 with stronger efficacy. As a result, less PAR2 were activated by KLK5 as the dose of SFTI-G increased, and the reduction of PAR2-dependent intracellular calcium mobilization was more obvious. These results suggested that SFTI-G was able to inhibit the activity of KLK5 in a does-dependent manner. 100 μM SFTI-G could effectively inhibit KLK5 and this concentration was used for further experiments. Therefore, SFTI-G inhibited the activity of exogenous recombinant KLK5, but the influence of SFTI-G on endogenously overexpressed KLK5 in live cells remained unknown and was further investigated.

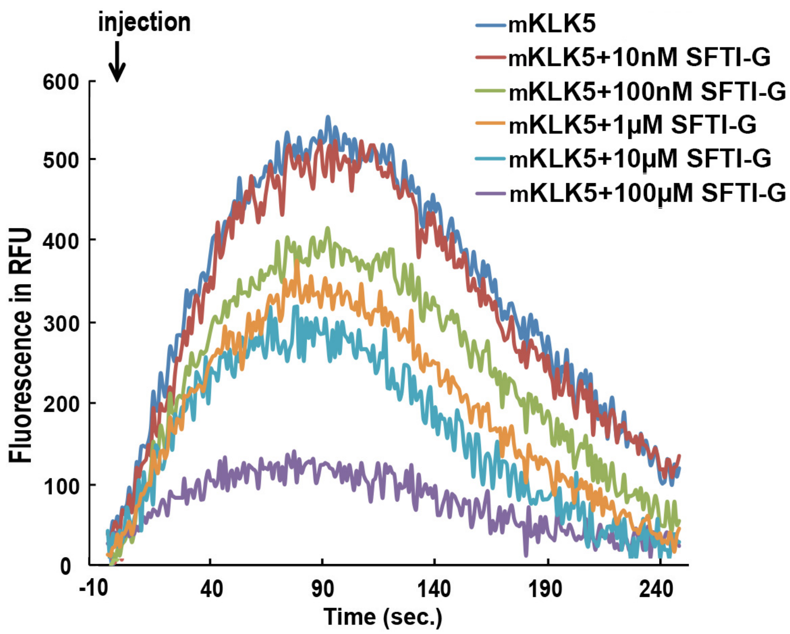

Figure 3.35. KLK5-stimulated intracellular calcium mobilization in normal keratinocytes was inhibited by SFTI-G.

Normal human keratinocytes were stimulated with the mixture of mKLK5 and SFTI-G at serial concentrations. Injection of mKLK5 alone triggered intracellular calcium mobilization in normal keratinocytes. The calcium mobilization was reduced in the presence of SFTI-G, and the reduction was more obvious as the concentration of SFTI-G increased, indicating SFTI-G could inhibit the activity of KLK5 in a does-dependent manner.

3.4.1.2 SFTI-G inhibited the activity of endogenously overexpressed KLK5 without affecting its expression

In order to study the influence of SFTI-G on endogenously overexpressed KLK5, KLK5-cells in culture were treated with SFTI-G. SFTI-G was used at 100 μM final concentration and the treatment of SFTI-G lasted for 48 hours. Untransduced cells and eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. No visible cell death was observed in all three types of cells treated with SFTI-G. Cell lysates and culture media were harvested 48 hours post-treatment and were subjected to further analysis.

SFTI-1 inhibits the activity of serine proteases by directly binding to its trypsin-like serine protease catalytic domain (Shariff et al 2014). As an SFTI-1-derived analogue, SFTI-G may exert its inhibitory activity in the same way as SFTI-1. As a result, the expression of KLK5 in KLK5-cells should not be affected by SFTI-G treatment. In order to confirm this, expression of KLK5 in the culture media of KLK5-cells treated with SFTI-G was examined using western blot analysis. Untransduced cells and eGFP-cells were treated in parallel. Untreated cells were used as controls. The levels of KLK5 in treated and untreated groups were compared using paired t-test and $p<0.05$ was considered as significant. The expression of KLK5 was detected in the culture media of KLK5-cells, whereas the levels of KLK5 in untransduced cells and eGFP-cells were not detectable. The intensity of KLK5 band remained unchanged in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells ($p>0.05$) (Figure 3.36). Therefore, the expression of KLK5 was not affected by SFTI-G treatment, suggesting that SFTI-G could inhibit the activity of KLK5 by interacting with its trypsin-like serine protease catalytic domain but not causing the degradation of KLK5.

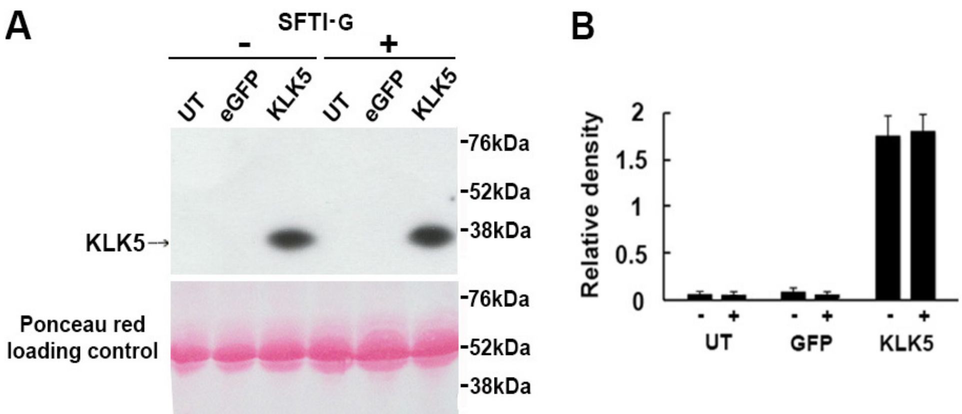

Figure 3.36. The expression of KLK5 was not affected by SFTI-G.
The expression of KLK5 in the cells with (+) or without (-) the treatment of SFTI-G was examined by western blot analysis. Ponceau red staining was used as loading control. Representative western blot out of three independent experiments (n=3) are shown (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). The expression of KLK5 was not affected in KLK5-cells after the treatment of SFTI-G. Error bars represent the standard error.

Previous experiments in this study indicated that consistent up-regulation of KLK5 in keratinocytes lead to impaired PAR2 function without affecting the expression of PAR2 (Section 3.2.3). Thus the expression of PAR2 in KLK5-cells treated with SFTI-G was examined using western blotting (Figure 3.37). Untransduced cells and eGFP-cells were treated with SFTI-G as well. Protein levels of PAR2 were quantified by densitometry and normalized to β-actin. The results revealed unchanged intensity of PAR2 blots in cells treated with SFTI-G compared to untreated cells, indicating the expression of PAR2 was not affected by SFTI-G treatment. Although the expression of PAR2 was not influenced by SFTI-G, the function of PAR2 in KLK5-cells treated with SFTI-G remained unclear and was further evaluated.

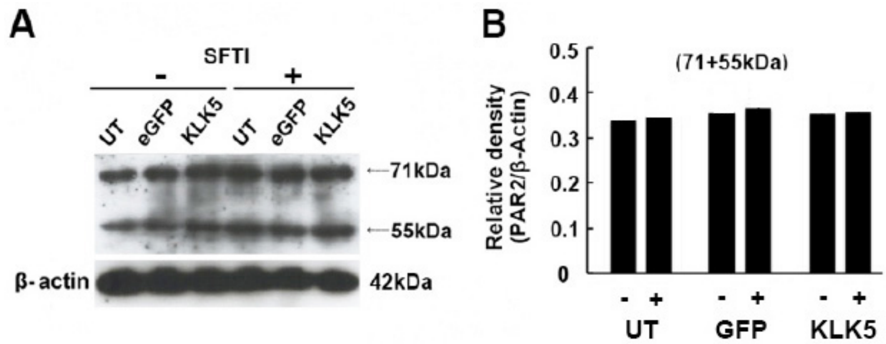

Figure 3.37. Expression of PAR2 in KLK5-cells treated with SFTI-G.
Expression of PAR2 in KLK5-cells treated with SFTI-G was evaluated by western blot analysis. Untransduced cells (UT) and eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. β-Actin was used as loading control. One representative experiment out of two is shown (A). Similar results were obtained from two independent experiments. Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). The expression of PAR2 in KLK5-cells was not affected by SFTI-G.

The level of PAR2-dependent intracellular calcium mobilization was reduced in keratinocytes consistently overexpressing KLK5, indicating the function of PAR2 was impaired in KLK5-cells (Section 3.2.3). This could be attributed to the desensitization of PAR2 under the repeated stimulation by consistently overexpressed KLK5. If the activity of endogenously overexpressed KLK5 could be inhibited by SFTI-G, the repeated stimulation of PAR2 by KLK5 might be prevented and the desensitization of PAR2 could be relieved. As a result, the impaired function of PAR2 should be restored and the level of PAR2-dependent intracellular calcium mobilization should be recovered in KLK5-cells followed by SFTI-G treatment. In order to confirm this, KLK5-cells were incubated with 100 μM SFTI-G overnight. The following day, treated KLK5-cells were stimulated with specific PAR2 agonist peptide (PAR2-AP) and

PAR2-dependent intracellular calcium mobilization was measured in time-course. eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls.

There was reduced level of PAR2-dependent intracellular calcium mobilization in untreated KLK5-cells compared to eGFP-cells, which was consistent with the previous results. Once KLK5-cells were treated with SFTI-G, the PAR2-dependent intracellular calcium mobilization was restored compared to untreated KLK5-cells (Figure 3.38). There was no obvious difference in PAR2-dependent intracellular calcium mobilization between treated and untreated eGFP-cells.

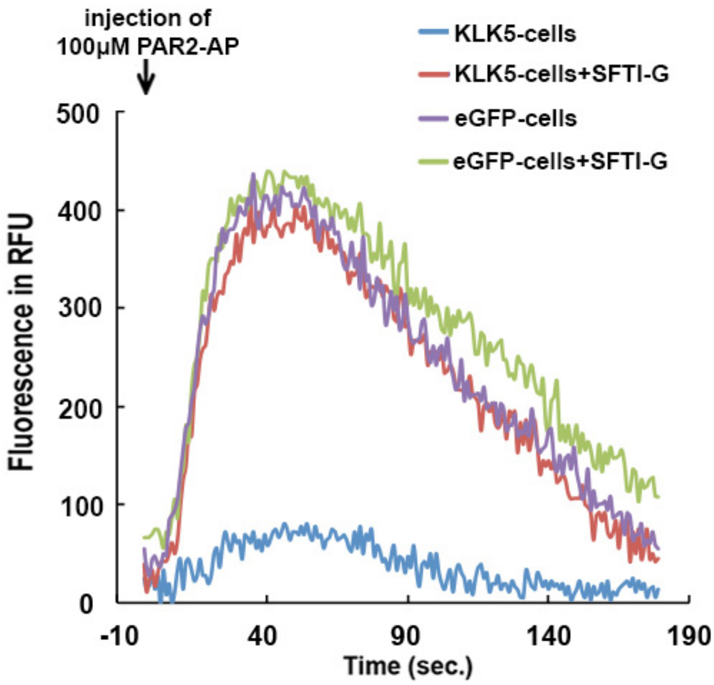

Figure 3.38. Recovered PAR2-dependent intracellular calcium mobilization in KLK5-cells treated with SFTI-G.

PAR2-dependent intracellular calcium mobilization in KLK5-cells treated with SFTI-G was examined. eGFP-cells were treated in parallel. Untreated cells were used as controls. All the cells were challenged with PAR2-AP. PAR2-dependant intracellular calcium mobilization was restored in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells. There was no obvious difference of PAR2-dependent intracellular calcium mobilization between treated and untreated eGFP-cells. Similar results were obtained from two independent experiments.

These results revealed that PAR2-dependent intracellular calcium mobilization was recovered in KLK5-cells treated with SFTI-G, indicating the impaired function of PAR2 was restored after SFTI-G treatment. It could be theorized that SFTI-G inhibited the up-regulated activity of endogenously overexpressed KLK5, which protected PAR2 from being repeatedly stimulated by consistently overexpressed KLK5. As a result, the desensitization of PAR2 was relieved and the function of PAR2 was restored, which caused recovered PAR2-dependent intracellular calcium mobilization in KLK5-cells treated with SFTI-G. The results indicated that the activity of endogenously overexpressed KLK5 could also be inhibited by SFTI-G. Further experiments were conducted to examine whether the abnormalities in relevant barrier-related proteins caused by overexpressed KLK5 could be reversed by SFTI-G treatment.

3.4.2 The abnormalities in barrier-related proteins caused by up-regulated KLK5 were reversed by SFTI-G treatment

3.4.2.1 Restored level of DSG1 in KLK5-cells treated with SFTI-G

Up-regulation of KLK5 resulted in over-degradation of corneodesmosomal protein DSG1 in keratinocytes (Section 3.2.3). If the activity of KLK5 could be inhibited by SFTI-G, the level of full-length DSG1 in KLK5-cells should be restored followed by the treatment of SFTI-G. In order to confirm this, the expression of DSG1 in KLK5-cells treated with SFTI-G was examined using western blot analysis. Untransduced cells and eGFP-cells were treated with SFTI-G in parallel. Untreated cells were used as controls. Protein levels of DSG1 were quantified by densitometry and normalized to β-actin.

The band of full-length DSG1 was detected at ~165kDa. The intensity of DSG1 band was decreased in untreated KLK5-cells compared to untransduced cells and eGFP-cells, which was consistent with the previous results. After SFTI-G treatment, the intensity of DSG1 band in KLK5-cells was increased (Figure 3.39), suggesting the restoration of full-length DSG1 in KLK5-cells treated with SFTI-G. The levels of DSG1 in both untransduced cells and eGFP-cells were not affected by SFTI-G treatment. These results indicated that SFTI-G could inhibit the uncontrolled activity of KLK5 in keratinocytes overexpressing KLK5, further preventing the over-degradation of DSG1 by unregulated KLK5.

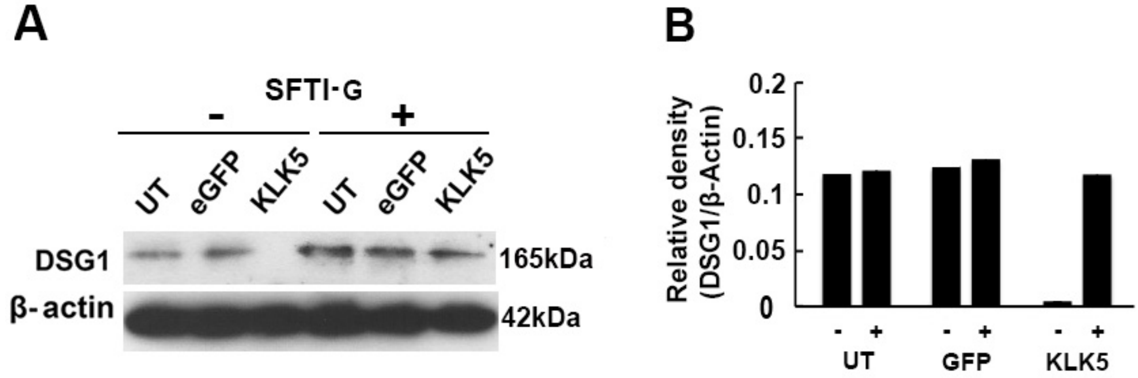

Figure 3.39. Restored level of DSG1 in KLK5-cells treated with SFTI-G.
Expression of DSG1 in KLK5-cells with (+) or (-) SFTI-G treatment was examined by western blotting. Untransduced cells (UT) and eGFP-cells were treated with SFTI-G as well. Untreated cells were used as controls. β-Actin was used as loading control. Representative western blot out of two independent experiments are shown (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). There was a restored level of full-length DSG1 in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells. The level of DSG1 was not changed in untransduced cells and eGFP-cells after the treatment of SFTI-G.

3.4.2.2 Reduced expressions of phospho-p53 and HSP60 in KLK5-cells followed by the treatment of SFTI-G

Overexpressed KLK5 also resulted in the up-regulation of phospho-p53 and HSP60 in keratinocytes (Section 3.3.3). If the activity of KLK5 could be inhibited by SFTI-G, the expressions of phospho-p53 and HSP60 in KLK5-cells should be reduced after the treatment of SFTI-G. However, inhibition of KLK5 should not affect the levels of other kinases. In order to confirm this, the expression of relevant kinases in KLK5 cells treated with SFTI-G was examined using human phospho-kinase array as previously described (Section 2.13). eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. The intensities of the blots were quantified using densitometry (Appendix 5). The expression of the target proteins was normalized to the reference proteins. Normalized protein levels of phospho-kinases were compared.

The results showed that the blot intensities of phospho-p53 and HSP60 were increased in untreated KLK5-cells compared to untransduced cells and eGFP-cells. Once KLK5-cells were treated with SFTI-G, the levels of phospho-p53 and HSP60 were reduced (Figure 3.40). The expression of phospho-p53 and HSP60 in eGFP-cells was not influenced by SFTI-G. In addition, the levels of other phospho-kinases were not affected by SFTI-G. These results indicated that the up-regulation of phospho-p53 and HSP60 in KLK5-cells was inhibited after SFTI-G treatment.

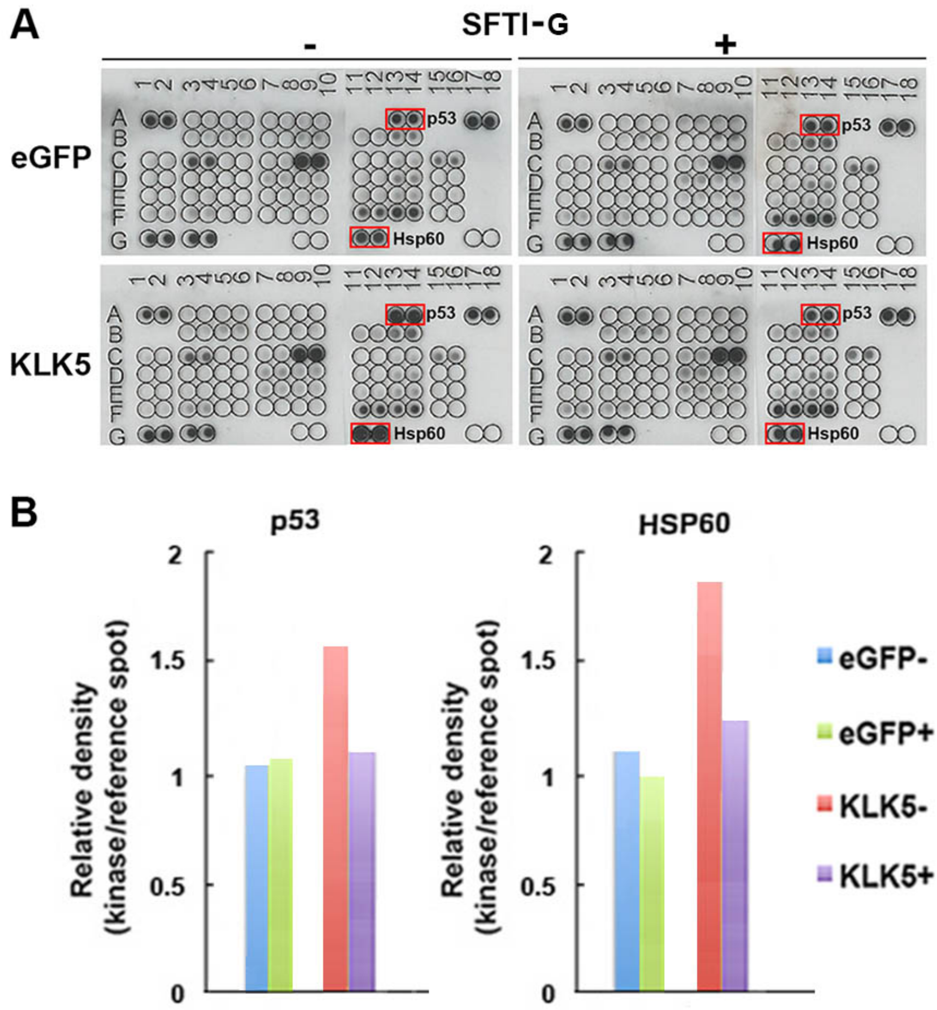

Figure 3.40. Reduced levels of phospho-p53 and HSP60 in KLK5-cells treated with SFTI-G.
The expression of 43 phospho-kinases and 2 related total proteins in KLK5-cells treated with SFTI-G was examined using human phospho-kinase array. eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. The expression of 22 phospho-kinases was detected in both cells (A). The expression of the target proteins was normalized to the reference protein (B). Reduced expression of p53 and HSP60 was found in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells. There were no differences of p53 and HSP60 between treated and untreated eGFP-cells.

The levels of phospho-p53 and HSP60 were further examined using western blot analysis. Untransduced cells and eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. The bands of phospho-p53 and HSP60 were detected at ~53kDa and ~60kDa respectively. The expression of phospho-p53 and HSP60 was increased in untreated KLK5 cells compared to untransduced cells and eGFP-cells. After SFTI-G treatment, the intensities of phospho-p53 and HSP60 bands in KLK5-cells were decreased (Figure 3.41&3.42), which was consistent with the results in kinase antibody array. The levels of phospho-p53 and HSP60 in untransduced cells and eGFP-cells were not affected by SFTI-G treatment. These results suggested that SFTI-G controlled the unregulated activity of KLK5 in KLK5-cells, further inhibiting the up-regulation of phospho-p53 and HSP60 caused by overexpressed KLK5. As increased phospho-p53 could be correlated with disorganized keratinocyte proliferation and differentiation, SFTI-G inhibited the up-regulation of phospho-p53 and might further correct the keratinocyte growth in KLK5-cells. In addition, overexpressed KLK5 might induce aberrant production of inflammatory cytokines in keratinocytes through up-regulating HSP60. Since the up-regulation of HSP60 was inhibited by SFTI-G treatment, the production of cytokines in KLK5-cells might be further affected.

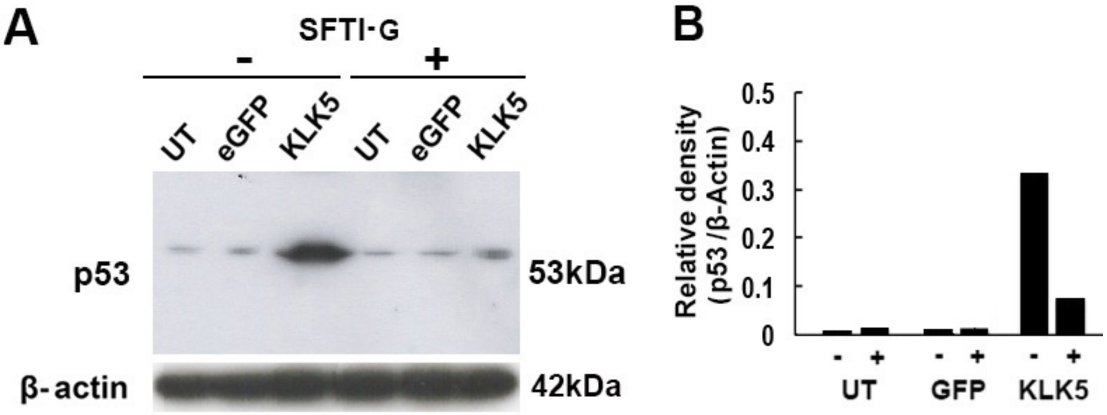

Figure 3.41. Decreased level of phospho-p53 in KLK5-cells treated with SFTI-G.
The expression of phospho-p53 in KLK5-cells treated with SFTI-G was examined using western blotting. β-Actin was used as loading control. One representative experiment out of two is shown (A). Similar results were obtained from two independent experiments. Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). Up-regulated expression of p53 was inhibited in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells. The level of p53 was not changed in untransduced cells (UT) and eGFP-cells after the treatment of SFTI-G.

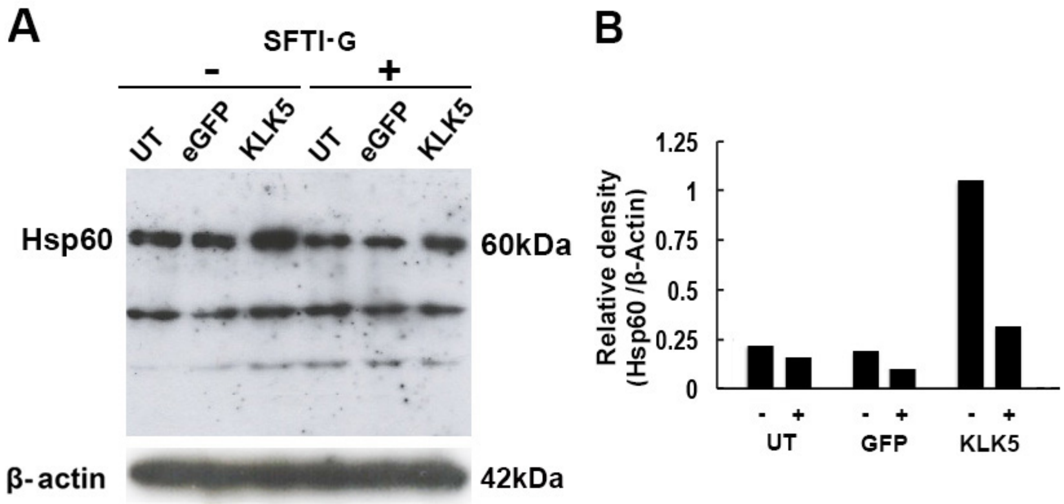

Figure 3.42. Reduced expression of HSP60 in KLK5-cells after the treatment of SFTI-G.
The expression of HSP60 in KLK5-cells treated with SFTI-G was examined using western blot analysis. β-Actin was used as loading control. Representative western blot out of two independent experiments are shown (A). Protein levels were quantified by densitometry as shown in the corresponding bar chart (B). Elevated expression of HSP60 was reduced in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells. The level of HSP60 was not changed in untransduced cells (UT) and eGFP-cells treated with SFTI-G.

3.4.2.3 Decreased secretion of IL-8, TSLP and IL-10 in KLK5-cells treated with SFTI-G

Previous results revealed that overexpressed KLK5 resulted in up-regulation of inflammatory cytokines IL-8, TSLP and IL-10 in keratinocytes (Section 3.3.5). If the activity of KLK5 could be inhibited by SFTI-G, the production of IL-8, TSLP and IL-10 in KLK5-cells should be reduced after the treatment of SFTI-G, but the levels of other cytokines should not be affected. In order to confirm this, the levels of cytokines in KLK5 cells treated with SFTI-G were examined using human cytokine array as previously described (Section 2.14). eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. The intensities of the blots were quantified using densitometry (Appendix 6). The expression of the target proteins was normalized to the reference proteins. Normalized protein levels of cytokines were compared (Section 2.14).

The results showed that the blot intensities of IL-8 and IL-10 were elevated in untreated KLK5-cells compared to untransduced cells and eGFP-cells. Once KLK5-cells were treated with SFTI-G, the levels of IL-8 and IL-10 were decreased (Figure 3.43). The production of IL-8 and IL-10 in eGFP-cells was not influenced by SFTI-G. In addition, the expression of other cytokines was not affected by SFTI-G. These results indicated that the increased levels of IL-8 and IL-10 in KLK5-cells were inhibited by SFTI-G treatment.

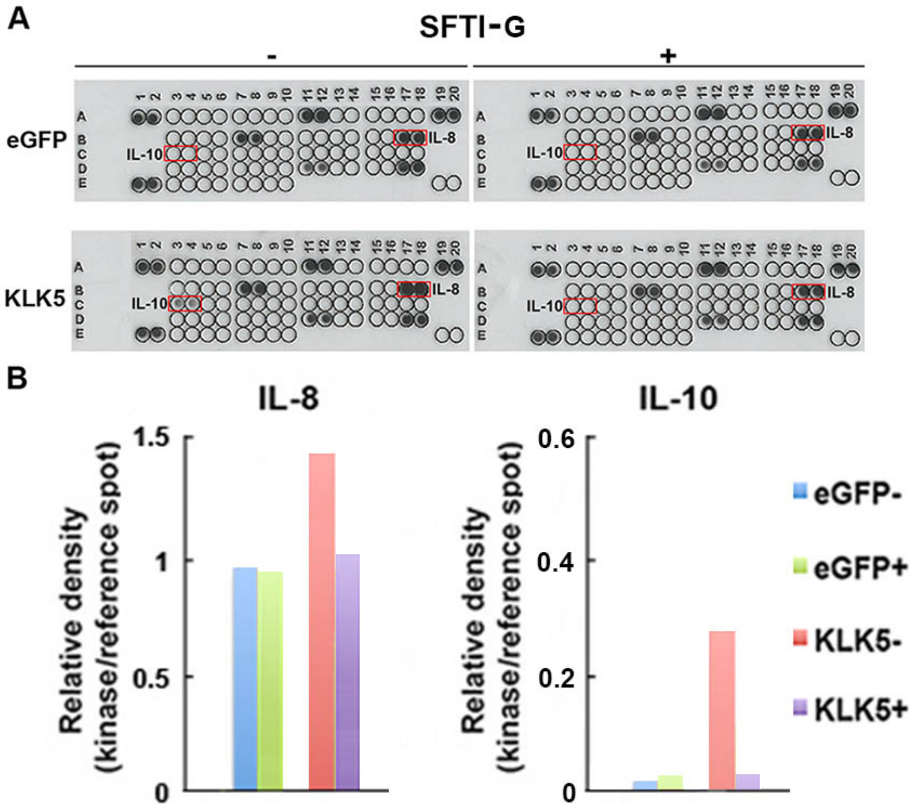

Figure 3.43. Reduced expression of IL-8 and IL-10 in KLK5-cells followed by the treatment of SFTI-G.

The expression of 36 cytokines in KLK5-cells treated with SFTI-G was examined using human cytokine antibody array. eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. The expressions of 6 cytokines were detected in both cells (A). Pixel density of each blot was calculated. The expression of the target proteins was normalized to the reference protein (B). Reduced expression of IL-8 and IL-10 was found in KLK5-cells treated with SFTI-G compared to untreated KLK5-cells. There was no difference of IL-8 and IL-10 between treated and untreated eGFP-cells.

The levels of IL-8, TSLP and IL-10 were further evaluated in samples from three independent experiments using ELISA. Untransduced cells and eGFP-cells were treated with SFTI-G as well. There was elevated production of these inflammatory cytokines in untreated KLK5-cells compared to untransduced cells and eGFP-cells. After SFTI-G treatment, the levels of IL-8, TSLP and IL-10 in KLK5-cells were significantly reduced ($p<0.05$) (Figure 3.44), which was consistent with the results in cytokine antibody array. The secretion of these inflammatory cytokines in untransduced cells and eGFP-cells was not affected by SFTI-G treatment. The results from both human cytokine antibody array and ELISA indicated that SFTI-G inhibited the activity of endogenously overexpressed KLK5, further preventing the up-regulation of IL-8, TSLP and IL-10 in KLK5-cells.

Previous findings in this study suggested that overexpression of KLK5 resulted in up-regulated HSP60 in keratinocytes, consequently leading to elevated secretion of IL-8, TSLP and IL-10 in KLK5-cells. Reduced level of HSP60 together with decreased production of these inflammatory cytokines was detected in KLK5-cells treated with SFTI-G. It was speculated that SFTI-G might control the activity of KLK5, further inhibiting the up-regulation of HSP60 caused by overexpressed KLK5 and reversing the aberrant secretion of IL-8, TSLP, IL-10 in KLK5-cells.

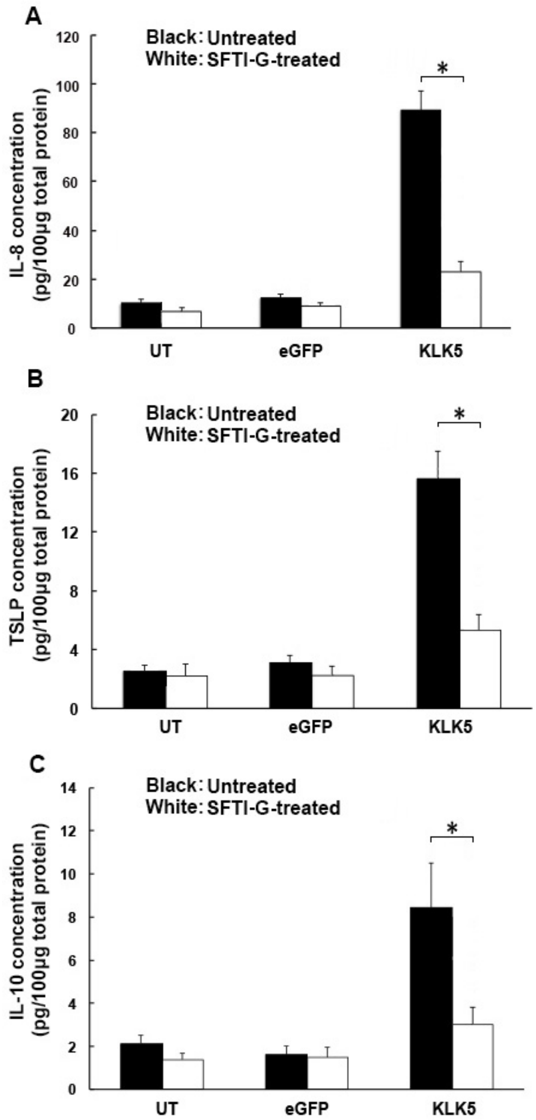

Figure 3.44. Reduced levels of IL-8, TSLP and IL-10 in KLK5-cells treated with SFTI-G.
*The expression of IL-8 (A), TSLP (B) and IL-10 (C) in KLK5-cells treated with SFTI-G was evaluated using ELISA. Untransduced cells (UT) and eGFP-cells were treated in parallel with KLK5-cells. Untreated cells were used as controls. Up-regulation of IL-8, TSLP and IL-10 was inhibited in KLK5-cells treated with SFTI-G but not in untreated KLK5-cells. The cytokines levels were not changed in untransduced cells and eGFP-cells after the treatement of SFTI-G. Error bars represent the standard error. Data represent mean±s.e.m. from three independent experiments (n=3). Error bars represent the standard error. * indicates results significant at p<0.05.*

3.4.3 Summary

SFTI-G was able to inhibit the activity of KLK5, further reversing the abnormalities in its down-stream molecules such as DSG1, p53, HSP60 and inflammatory cytokines, which are key regulators of the epidermal barrier function. SFTI-G may interact with KLK5 through binding to its trypsin-like serine protease catalytic domain rather than causing the degradation of KLK5. These results suggested that inhibition of up-regulated KLK5 could prevent the dysregulations of these barrier-related proteins, further contributing to the restoration of skin barrier.

The results in this study serve as a useful starting point for the development of a potent and selective inhibitory peptides towards KLK5. Therefore, SFTI-1 could be used as a scaffold to generate the specific inhibitor for KLK5. Application of the potential KLK5-specific inhibitor may inhibit the up-regulation of KLK5 in AD, further reversing the abnormalities in relevant barrier-related proteins and improving the function of epidermal barrier. This may prevent the development of AD and could be a promising therapeutic intervention for this skin disorder.

CHAPTER 4: DISCUSSION

4.1 Discussion

This work sought to investigate the contribution of up-regulated KLK5 to epidermal barrier dysfunction in AD and validate the inhibitory activity of SFTI-1 analogue towards KLK5. Several noteworthy findings from this study will be discussed.

Epidermal barrier defect in AD patients

The formation and function of epidermal barrier can be evaluated using noninvasive techniques including tape strips and invasive methods such as skin biopsy analysis. Tape stripping has been used to evaluate the barrier function in AD skin (Komatsu et al. 2007b; Voegeli et al. 2009, 2011). Although tape stripping is a relatively milder technique especially for children, it still has some limitations. For instance, tape strips are able to remove the outermost cells from the epidermis, which are corneocytes. As a result, only those molecules that are abundantly expressed in the cornified layer can be evaluated using this technique. Although many key components of epidermal barrier can be detected in corneocytes, the formation of skin barrier is a dynamic process that occurs during the transition from granular keratinocytes to corneocytes. Some barrier-related proteins such as DSG1 and FLG are already expressed in the granular layer, but the levels of these proteins cannot be properly measured using tape strips. In comparison, skin biopsy is a powerful tool for morphological examination of the entire epidermal barrier, and it can reflect the expression of barrier-related proteins more precisely. Therefore, biopsy analysis can help the dermatologists to identify the skin conditions and make diagnosis for skin diseases. However, since no specific histological features can be used to distinguish AD from other skin diseases with similar symptoms and most of the affected individuals are children, biopsies are not routinely taken from AD patients for diagnosis.

As AD is characterized by primary skin barrier dysfunction, biopsy analysis is necessary for the investigation of barrier defect in AD skin. Studies using skin biopsies from AD patients have already been performed by other research groups. For example, to investigate the localization of KLKs in AD skin, biopsy from one AD patient has been taken and subjected to immunostaining (Stefansson et al. 2008). In addition, to examine the expressions of antimicrobial protein and keratins in AD, skin biopsies obtained from four and seven AD patients were analyzed by immunohistochemical staining respectively (Komatsu et al. 2005; Gläser et al. 2009). Levels of barrier-related proteins including keratins were evaluated in skin biopsies obtained from twenty-five AD patients (Jensen et al. 2004). The staining results in one patient can well indicate the distribution of barrier proteins in AD skin. However, these findings could not reflect the protein levels in AD patients accurately, since the variation among different individuals is considerable. Therefore, in order to evaluate the protein expression in AD skin, average protein levels should be examined in biopsies obtained from a group of AD patients, which can reflect the trend of protein expression in AD skin and represent the real situation in AD more precisely. In this project, skin biopsies obtained from five AD patients and five age-matched healthy controls were subjected to histological and immunohistological analysis.

The levels of barrier-related proteins including KLK5, DSG1, PAR2 and FLG in skin biopsies were evaluated by immunostaining, and the intensities of staining were quantified by computer-assisted image analysis. This quantification method using Image Pro-Plus has been previously used for the visual assessment of staining in specimens including skin sections, which is considered to be accurate and reliable (Allen & Southern 2002; Wang et al. 2009). Aberrant epidermal morphology and dysregulations of skin barrier proteins were detected in AD patients. However, the severities of disturbed skin morphology and the impairments in barrier-related proteins varied among different AD individuals. For example, the extents of acanthosis and superficial dermal perivascular inflammatory infiltrate were more severe in the lesional skin from some AD patients than the others. In addition, up-regulation of KLK5 was more obvious in the lesional sites from some AD patients. Furthermore, deficiencies of barrier structural proteins DSG1 and FLG were more severe in the lesional skin of some affected individuals than the others. In addition, impaired epidermal morphology and disturbed expression of barrier-related proteins were already found in the healthy-looking "nonlesional" skin obtained from some AD patients, although far less prominent than those detected in the lesional skin. These findings were consistent with the previous reports, which reveal that clinically nonlesional sites of AD also display skin barrier abnormalities (Seidenari and Giusti 1995; Jensen et al. 2004; Bieber 2008). These variations could be attributed to various disease severities in different individuals. The disease phenotypes might already exist in the nonlesional skin of patients with more severe AD but not in those with milder AD. Despite the various protein levels among different AD patients, our results can reflect the trend of barrier-related proteins in AD skin. These preliminary findings provide indications for further investigation.

Possible influences of up-regulated KLK5 in AD skin

The results suggested that KLK5 was up-regulated in AD skin especially in the lesional sites, which is consistent with previously reported increased expression and enhanced activity of KLK5 in AD (Komatsu et al. 2005, 2007b; Voegeli et al. 2009). Interestingly, up-regulated KLK5 has also been reported in other inflammatory skin disorders including psoriasis and NS (Descargues et al. 2005, 2006; Hachem et al. 2006b; Komatsu et al. 2007a). Similar as AD, these diseases were also characterized with epidermal barrier dysfunction. Since the etiologies of these skin disorders are different, up-regulated KLK5 may not be the initial cause of these diseases. However, KLK5 up-regulation could be an important "accelerator", which can exacerbate the epidermal barrier defect and trigger the development of these skin disorders. Therefore, it was speculated that up-regulation of KLK5 could play a key role in barrier dysfunction in skin diseases such as AD.

However, the causes of KLK5 up-regulation in AD are unclear. As AD is a chronic skin disorder, many genetic and environmental factors could lead to persistent epidermal barrier defect in AD, consequently resulting in consistently up-regulated KLK5. For example, variants in LEKTI gene can result in lack of inhibition against KLK5, subsequently leading to up-regulated activity of KLK5 in AD skin (Fortugno et al. 2012). In addition, variations of FLG gene and overuse of soap could both exacerbate epidermal barrier defect and lead to increased pH in the epidermis of AD skin (Cork et al. 2009). As a result, a sustained increase in pH leads to hyperactivity of some epidermal proteases such as KLK5, which have optimum activity at slightly alkaline pH.

Up-regulated KLK5 could subsequently contribute to the skin barrier dysfunction in AD through its downstream molecules. Since KLK5 is involved in the proteolytic processing of epidermal barrier structural proteins DSG1 and FLG, up-regulation of KLK5 could result in deficiencies of these molecules in AD. Unopposed activity of KLK5 induces over-degradation of corneodesmosomes and consequently leads to impaired epidermal barrier integrity (Yang et al. 2004; Descargues et al. 2005; Wang et al. 2014). Furthermore, the E420K variant in SPINK5 gene, one of the most common polymorphisms in AD and NS, results in the decreased formation of LEKTI D6-D9 fragment, which displays strong inhibition against KLK5 and KLK5-mediated cleavage on DSG1, subsequently leading to over-degradation of DSG1 (Fortugno et al. 2012). Results in our study showed reduced level of DSG1 in AD skin, indicating up-regulated

KLK5 activity in AD could result in the over cleavage of DSG1 and subsequently lead to deficient skin barrier integrity. In addition, up-regulated KLK5 is capable of inducing the hyperactivity of ELA2, which degrades both pro-FLG and FLG monomers (Bonnart et al. 2010). Unopposed activity of KLK5 in AD might also promote the activation of ELA2, consequently leading to the misprocessing and deficiency of FLG. However, variations within FLG gene could also result in reduced expression of FLG in AD (Barker et al. 2007; Morar et al. 2007; Brown et al. 2008; Ekelund et al. 2008; De Benedetto et al. 2008). Although our results showed reduction of FLG in AD skin, the AD patients recruited in our study have not been genotyped for FLG mutations, thus the deficiency of FLG might also be attributed to variants in FLG gene. Therefore, the association between up-regulated KLK5 and decreased level of FLG in AD could not be concluded.

Keratinocytes overexpressing KLK5 as a viable model to study the roles of KLK5 in AD

As AD is a multifactorial disease, it is difficult to generate a model of AD, which can exhibit all the features of this skin disorder. However, experimental models have been generated to dissect the roles of some key factors involved in the onset and development of AD. Until now, a number of models have been established. The first model of AD (Nc/Nga mice) has been reported in 1997. These mice develop AD-like skin changes spontaneously after being repeatedly exposed to various environmental aeroallergens (Matsuda et al. 1997). Nc/Nga mice show skin barrier manifestations with increased TEWL and impaired ceramide metabolism, all of which might predispose these mice to the development of AD (Aioi et al. 2001). In addition, models were developed using mechanical injury, which mimic the skin barrier deficiency in AD. Repeated tape-stripping followed by epicutanous sensitization using ovalbumin on the skin could induce the release of IL-10 from keratinocytes and promote the Th2 response (Laouini et al. 2003a, b). Furthermore, as AD is characterised Th2 immune response, models mimicking the immune dysregulation in AD have been generated. These models dissect the roles of immune regulators including cytokines in the development of AD. For instance, transgenic mice overexpressing Th2 cytokine IL-4 display features of human AD, including acanthosis, mild spongiosis and dermal infiltration (Chan et al. 2001). Overexpression of pro-Th2 cytokine TSLP also leads to the skin lesions similar to those observed in human AD, such as acanthosis, spongiosis and dermal infiltration. The lesions of TSLP transgenic mice also exhibit up-regulated expression of Th2 cytokines (Yoo et al. 2005).

Pathogenesis of AD involves both skin barrier defect and immune dysregulation. Although these established models allow in-depth investigation of critical mediators especially immune regulators in AD, the mechanism underlying epidermal barrier defect, which is the primary cause of AD, remains largely unexplored. KLKs are key regulators of skin barrier function and up-regulation of major epidermal KLKs such as KLK5 and KLK7 has been reported in AD. Furthermore, overexpression of KLK7 in transgenic mice leads to the development of AD-like skin lesions characterized by increased epidermal thickness and dermal inflammation (Hansson et al. 2002). Recently, transgenic mice model overexpressing KLK5 has been established. These mice display characteristic features of AD, such as increased activity of epidermal proteases and up-regulation of inflammatory cytokines including TSLP (Furio et al. 2014). These findings indicated that up-regulated KLKs including KLK5 could contribute to the skin barrier dysfunction in AD. Despite the well-established regulatory function of KLK5 in epidermal barrier through its downstream molecules, the influences of up-regulated KLK5 on barrier defect in AD is not well defined and needs to be further investigated. As the pathogenesis of AD is complicated, dysregulations of numerous molecules can contribute to the impaired integrity and defective function of epidermal barrier in this skin disorder. In order to dissect KLK5 from other causes of AD and investigate the contribution of KLK5 up-regulation alone to the skin barrier defect, keratinocytes ectopically overexpressing KLK5 were generated. This cell model was further used to study the influences of consistent KLK5 up-regulation on the function of epidermal barrier.

Human keratinocytes overexpressing KLK5 were successfully generated using lentiviral vector. Transfer of eGFP reporter genes, either alone or in combination with KLK5 gene, was readily achieved by exposure of cultured human keratinocytes to a single round of virus infection. The proportion of transduced cells confirmed by the expression of eGFP was similar in both cases. The results indicated that KLK5 was overexpressed, secreted and activated in KLK5-cells, which could mimic the up-regulation of KLK5 in the AD skin. As our construct expressed KLK5 as a proprotease, these findings suggested that ectopically overexpressed pro-KLK5 was activated *in vitro*. Previous studies have reported that after being secreted into the extracellular compartments, pro-

KLK5 could be processed into the active form through proteolytic cleavage. Besides of auto-activation, pro-KLK5 could be activated by KLK14 *in vitro* (Brattsand et al., 2005). Recently, it has been revealed that other serine proteases such as matriptase and mesotrypsin can also activate pro-KLK5 *in vitro* (Sales et al., 2010; Miyai et al. 2014). It was speculated that overexpressed pro-KLK5 in keratinocytes could be activated by other proteases or through self-activation, which may result in the up-regulated KLK5 activity in KLK5-cells.

In order to further investigate the influences of up-regulated KLK5 on the epidermal barrier, *in vitro* OTCs were generated using primary human keratinocytes overexpressing KLK5. Adaptation of OTC techniques, also known as rafts, provided the tool to evaluate the keratinocyte growth and epidermal formation *in vitro*. However, many factors could affect the three-dimensional skin-equivalent culture, such as passages of the primary keratinocytes, replenishment of the fresh culture media and maintenance of the air-liquid interface. It was difficult to generate fully stratified cultures with low reproducibility between different batches if these factors are not properly controlled. Therefore, the experiments were repeated many times to improve the consistency of different cultures. Furthermore, previously established bioengineered skin-humanized mouse model was also used to study the influences of overexpressed KLK5 *in vivo* (Di Nunzio et al. 2008). Successful skin grafts rely on various factors, such as the proportions of complex components in the matrix, confluence of the keratinocytes before grafting, thickness of the bioengineered skin constructs and age of the mice. In order to improve the successful rate for the grafting, these experimental conditions were optimized and strictly controlled during the generation of skin grafts.

Overexpressed KLK5 in human keratinocytes was able to induce AD-like histological features and skin barrier impairments in both *in vitro* and *in vivo* experiments. Furthermore, overexpression of KLK5 showed potential to induce disorganized keratinocyte growth and overexpression of TSLP and IL-10, which have also been previously observed in AD skin (Ohmen et al. 1995; Howell et al. 2005; Proksch et al. 2006). These findings suggested that up-regulated KLK5 could contribute to these epidermal barrier impairments in AD. Therefore, our KLK5 overexpression cells revealed the importance of KLK5 up-regulation in AD pathogenesis. Although this cell model may not completely reflect all the hallmarks of AD, it can be further used as a viable model to study the influences of up-regulated KLK5 on epidermal barrier defect in AD.

Consistently overexpressed KLK5 induced impaired PAR2 function

As KLK5 is an activator of PAR2 in human keratinocytes (Briot et al. 2004), consistent up-regulation of KLK5 may also affect the function of PAR2 in AD. Although unchanged expression of PAR2 was found in KLK5-cells, the influences of consistently up-regulated KLK5 on PAR2 function remained unknown. Upon being activated, PAR2 binds to G proteins and triggers the intracellular calcium mobilization, further exerting various biological functions in the cells (Kawabata 2002). Therefore, the level of PAR2-dependent intracellular calcium mobilization can reflect the function of PAR2. Our results showed that single stimulation by PAR2-AP or rKLK5 caused PAR2-dependent intracellular calcium mobilization in normal keratinocytes. The calcium response was relatively slower in cells challenged with rKLK5 compared to those stimulated with PAR2-AP. This could be due to the different activating mechanisms of PAR2 mediated by PAR2-AP and KLK5, as PAR2-AP triggers the activation of PAR2 through direct binding, while rKLK5 activates the receptor via proteolytic cleavage. However, there was reduced PAR2-dependent intracellular calcium mobilization in keratinocytes overexpressing KLK5, suggesting the function of PAR2 was impaired under the consistent stimulation by overexpressed KLK5.

We speculated PAR2 desensitization could be responsible for the impaired PAR2 function in KLK5-cells. Previous studies revealed that exposure to PAR2 stimuli at a supramaximal concentration or repeated stimulation leads to the desensitization of PAR2 (Böhm et al. 1996; Dery et al. 1998; Oikonomopoulou et al. 2006). When PAR2 undergoes desensitization, G protein receptor kinase and b-arrestin mediate the phosphorylation of activated PAR2 on its C-terminus, consequently disrupt the interaction between PAR2 and G proteins in this domain (Böhm et al. 1996; Dery et al. 1998). This can trigger the uncoupling of activated PAR2 from G proteins, resulting in the termination of PAR2-mediated signal (Böhm et al. 1996; Dery et al. 1998). Therefore, desensitization of PAR2 protects the cells from uncontrolled stimulation by its stimuli, which renders the cells irresponsive to further stimulation of PAR2 stimuli for a considerable period of time, until the plasma membrane is replenished with intact PAR2 through a regulatory mechanism called resensitization (Böhm et al. 1996). During the resensitization of PAR2, the level of membrane-bound intact PAR2 is replenished through synthesis of new receptors and mobilization of intracellular pools of the receptor (Böhm et al. 1996). As a result, the responsiveness of PAR2 to its stimuli can be recovered. It could be theorized that consistent overexpression of KLK5 in keratinocytes resulted in continuous stimulation on PAR2, further triggering the desensitization of PAR2, which can consequently induce aberrant termination of PAR2-mediated signal transduction. Although PAR2 desensitization could be partially compensated by resensitization, the level of membrane-bound intact PAR2 may not be completely restored. As a result, the function of PAR2 was diminished in KLK5-cells, and the level of PAR2-dependent intracellular calcium mobilization was decreased. If PAR2 is desensitized in KLK5-cells, the level of PAR2 phosphorylated on C-terminus should be increased. However, this was not confirmed in this study, since we currently lack antibodies to this form of PAR2.

As a chronic skin disorder, many factors such as SPINK5 and FLG gene polymorphisms and overuse of soap could result in consistently up-regulated KLK5 in AD. In addition, overexpression of KLK5 was already observed in the nonlesional sites of some AD patients, suggesting the up-regulation of KLK5 in AD skin was consistent. As a result, PAR2 could be desensitized under the continuous stimulation of up-regulated KLK5 in AD. Furthermore, our results showed that PAR2-dependent intracellular calcium mobilization was recovered in KLK5-cells treated with SFTI-G, indicating the impaired function of PAR2 was restored after SFTI-G treatment. It could be theorized that SFTI-G inhibited the uncontrolled activity of KLK5, which protected PAR2 from being repeatedly stimulated by consistently overexpressed KLK5. Then the desensitization of PAR2 was relieved and the function of PAR2 was restored, further causing the recovered PAR2-dependent intracellular calcium mobilization in KLK5-cells. These results confirmed that PAR2 desensitization could be induced by consistently up-regulated KLK5, which was reversed by inhibiting unopposed KLK5 activity.

Up-regulated KLK5 may contribute to disorganized keratinocyte growth

Apart from AD-like epidermal barrier disruptions, impaired suprabasal morphology and poorly differentiated appearance of keratinocytes were also found in OTCs and skin grafts generated from KLK5-cells, suggesting up-regulation of KLK5 possibly interfered keratinocyte proliferation and differentiation. Therefore, human phospho–kinase antibody array that contains numerous kinases relevant to keratinocyte growth was performed. The levels of 43 phospho-kinases were examined in KLK5-cells. The results showed that overexpression of KLK5 induced up-regulation of phospho-p53^{S392}

in keratinocytes. In comparison, the levels of p53 phosphorylated on S15 and S46 were unchanged in KLK5-cells (Appendix 3).

p53 is a transcription factor regulating the expression of numerous growth-related genes. Activation and stabilization of p53 is mediated through phosphorylation (Jimenez et al. 1999; Maclaine & Hupp 2009). There are eighteen different phosphorylation sites in p53, phosphorylation may occur at different sites in response to certain stress (Jimenez et al. 1999; Maclaine & Hupp 2009). The phosphorylation of p53 is regulated by numerous kinases, including protein kinases belongs to the MAPK family such as ataxia telangiectasia mutated (ATM), checkpoint kinases 1 and/or 2 (CHK1/2), cyclin-dependent kinases (CDKs) and casein kinase 2 (CK2) (Claudio et al. 2006; Jimenez et al. 1999; Maclaine & Hupp 2009). CDKs and CK2 are involved in the phosphorylation of p53 on S392 (Claudio et al. 2006). Overexpressed KLK5 could trigger the phosphorylation of p53 on S392 through activation of CDKs/CK2. In addition, our results showed the up-regulation of phospho-p53^{S392} in KLK5-cells was inhibited after SFTI-G treatment. These findings confirmed that the increased level of phospho-p53^{S392} was caused by overexpressed KLK5, which could be reversed through inhibition of up-regulated KLK5 activity.

Previous study reported that maximum expression of p53 was found in proliferating keratinocytes (Dazard et al. 2000), where phospho-p53^{S392} could be the major form of p53 that functions to ensure the correct control of cell cycle and cell division. In this study, down-regulation of p53 and up-regulation of MDM2 were also detected in keratinocytes transitioning from proliferation to differentiation (Dazard et al. 2000). MDM2 is one of the transcriptional targets of p53, which can inactivate p53 protein by forming the complex with it in a negative feedback loop, consequently triggering the degradation of p53 by the proteasome through ubiquitin system and in turn down-regulating p53 (Piette et al. 1997; Freedman et al. 1999). These results suggested that the switch from p53 to MDM2 occurs during the transformation of keratinocytes from proliferation to differentiation, and the levels of p53 and MDM2 are delicately controlled during the growth of keratinocytes. However, up-regulated phospho-p53^{S392} may correlate with imbalanced keratinocyte proliferation and differentiation in KLK5-cells, which could consequently result in disrupted skin barrier formation. In order to confirm this, the level of MDM2 and expression of proliferation/differentiation markers in KLK5-cells can be evaluated in future experiments.

AD is characterized with disrupted keratinocyte proliferation and differentiation. Enlarged expression and increased levels of proliferation-associated proteins including keratin 5 and Ki-67 are detected in both AD nonlesional and lesional skin (Proksch et al. 2006). In addition, diffused staining zone and reduced expression of differentiation-related molecules, such as FLG, involucrin and keratin 10, are also found in AD skin especially in the lesional sites (Proksch et al. 2006). Furthermore, disorganized keratinocyte growth is also found in previously established models of AD. For instance, the lesions of Nc/Nga mice exhibit skin manifestations including parakeratosis, suggesting the disturbed differentiation of keratinocytes in this AD animal model (Matsumoto et al. 1997). In addition, overexpression of KLK7 in transgenic mice leads to the development of AD-like skin impairments characterized by marked acanthosis, indicating an increased cell number resulted from up-regulated keratinocyte proliferation (Hansson et al. 2002). These findings indicated that AD-like lesion is characterized with disrupted keratinocyte proliferation and differentiation, which can lead to impaired epidermal barrier formation and exacerbate the barrier dysfunction in AD skin. Overexpressed KLK5 could contribute to disorganized keratinocyte growth in AD by up-regulating phospho-p53^{S392}. Therefore, our KLK5-cells could be used as a viable model to study the influences of up-regulated KLK5 on keratinocyte growth, which may be part of KLK5-triggered vicious cycle of skin barrier defect in AD.

Overexpressed KLK5 could trigger the up-regulation of proinflammatory cytokines in keratinocytes

The results also showed increased level of HSP60 in KLK5-cells. HSP60 exhibits multiple regulatory functions in various cellular signalling pathways. Like other HSPs, HSP60 is up-regulated when cells are exposed to cellular stresses such as elevated temperature, which can prevent or correct the protein misfolding caused by heat. Apart from its typical role as a heat shock protein, HSP60 is also a mitochondrial chaperonin, which involves in the transportation and maintenance of mitochondrial proteins (Vargas-Parada et al 2001; Cappello et al. 2006; Urushibara et al. 2007). In addition, HSP60 plays a potential role in a “danger signal cascade” in the immune response (Pockley 2001). It can stimulate the secretion of a wide range of inflammatory cytokines, consequently promoting the inflammation in numerous types of cells (Srivastava 2002; Henderson & Pockley 2010; Kang et al. 2013; Tian et al. 2013). Our results suggested that overexpression of KLK5 resulted in increased levels of inflammatory cytokines IL-8, TSLP and IL-10 in keratinocytes potentially though up-regulating HSP60. Furthermore, overexpression of HSP60 and inflammatory cytokines were inhibited in KLK5-cells treated with SFTI-G, confirming that the up-regulation of these molecules was induced by overexpressed KLK5, which could be inhibited by controlling the unopposed KLK5 activity.

AD is characterized with Th2-biased immune response and aberrant cytokine production. For example, increased levels of TSLP and IL-10 were detected in the skin of AD patients (Ohmen et al. 1995; Soumelis et al. 2002; Howell et al. 2005; Sano et al. 2013). In addition, immune dysregulation has also been revealed in previously established AD models. Topical application of the recombinant house dust mite allergens or epicutanous sensitization triggered by ovalbumin can induce AD-like skin inflammation characterized with Th2 immune response (Huang et al. 2003; Laouini et al. 2003). In addition, transgenic mice overexpressing TSLP exhibit dermal infiltration and a Th2 cell profile with the up-regulation of Th2 cytokines, which are also observed in AD skin (Yoo et al. 2005). Although these findings revealed the immune dysfunction in AD-like lesions, the mechanisms underlying the cytokine imbalance in AD are not completely elucidated.

Up-regulation of KLK5 could contribute to the increased levels of TSLP and IL-10 in AD. TSLP can initiate the differentiation of Th2 cells and induce Th2 cytokine secretion, and it is involved in the Th2 mediated allergic inflammation including skin disorders (Liu et al. 2007; Roan et al. 2012; Bell et al. 2013; Jang et al. 2013). IL-10 is also able to promote the Th2 response in a murine model of allergic dermatitis (Laouini et al. 2003a). Up-regulation of TSLP and IL-10 could subsequently contribute to the skewed Th2 immune response in AD. As a result, up-regulated Th2 cytokines may lead to reduced level of lipid production and decreased amount of corneodesmosomes in the epidermis, consequently abrogating the intercellular cohesion and exacerbating skin barrier defect in AD (Sawada et al. 2012; Hatano et al. 2013). Furthermore, up-regulated IL-10 could also be responsible for the reduced levels of AMPs such as LL-37 and b-defensin in AD (Howell et al. 2005). Deficient levels of AMPs leave the skin with a lack of protection against the exogenous pathogens. Overexpressed KLK5 could contribute to the deficiency of AMPs in AD by up-regulating IL-10, consequently favoring the invasion of allergens/pathogens, which can exacerbate the skin barrier dysfunction and promote the development of AD (Otto 2004; Clarke et al. 2007; Roelandt et al. 2008; Hirasawa et al. 2010). In addition, overexpressed KLK5 caused up-regulation of IL-8 in keratinocytes. Keratinocyte-derived IL-8 functions as a chemoattractant to cause the migration of human neutrophils and T cells toward the site of inflammation (Barker et al. 1991). Since AD is characterised by dermal perivascular inflammatory infiltrate, KLK5 overexpression may trigger the recruitment of neutrophils/T cells into the epidermis by increasing the level of IL-8, consequently contributing to the inflammatory infiltrate in AD.

Since the influences of other causes of AD have been eliminated in our KLK5 overexpression model, the increased levels of IL-8, TSLP and IL-10 are attributed to up-regulated KLK5. KLK5 up-regulation could contribute to the immune dysregulation in AD by increasing the levels of these proinflammatory cytokines, consequently aggravating the barrier dysfunction. However, apart from HSP60, overexpressed KLK5 may induce the up-regulation of IL-8, TSLP, IL-10 through other regulators, which were not evaluated in our study. Therefore, whether this potential role of KLK5-mediated signalling in inflammatory response relies on HSP60 remained uncertain. In order to further confirm that up-regulated KLK5 leads to the overexpression of these cytokines in keratinocytes in an HSP60-dependent manner, KLK5-cells could be treated with potential HSP60 inhibitors such as mizoribine in future experiments. Mizoribine bounds to HSP60 directly and in turn inhibits the function of HSP60 (Itoh et al. 1999). The levels of IL-8, TSLP and IL-10 can be examined in KLK5-cells treated with mizoribine to check whether the up-regulation of these inflammatory cytokines is inhibited by blocking the function of HSP60.

Prospect of potential KLK5-specific inhibitor in AD therapy

Up-regulated KLK5 not only induced over-degradation of DSG1, but also showed potential to cause disrupted cell growth and aberrant cytokine production in keratinocytes, possibly through p53 and HSP60. These abnormalities could result in impaired skin barrier formation and trigger the immune dysregulation, consequently exacerbating the barrier dysfunction and promoting the development of AD. Thus KLK5 appeared to be a potential target for the treatment of AD. As barrier defect is the primary cause of AD, therapeutic approaches should be first directed at improvement of the skin barrier. Inhibition of up-regulated KLK5 may restore the epidermal barrier function, making it a promising therapeutic intervention for AD.

Sunflower trypsin inhibitor (SFTI-1) is a 14 amino acid cyclic peptide (Gly-Arg-Cys-

Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp) extracted from the sunflower seeds. It exhibits exceptionally potent trypsin-inhibitory activity. SFTI-1 and SFTI-1-derived analogues are known to act upon a wide range of serine proteases including cathepsin G7, matriptase and KLKs. Mutation of selected SFTI-1 residues can improve its inhibitory specificity towards certain substrates (Long et al. 2001; Swedberg et al. 2009; De Veer et al. 2013; Tan et al. 2013). In addition, SFTI-1 possesses highly stable fold due to its cyclic backbone and a cross-bracing disulphide, thus it is considered as a promising peptide-based drug bioscaffold. Insertion of bioactive peptide sequences into the circular scaffold of SFTI-1 has been shown to improve its *in vivo* stability (Chan et al. 2011). Due to its size, activity and stability, SFTI-1 and its analogues are attractive therapeutic drug candidates.

In this study, Dr. Macmillan's group at UCL Chemistry Department demonstrated that circular peptides could be prepared via a native chemical ligation-type process. This strategy was utilised for the first time to produce the analogues of SFTI-1. Ile10 was substituted with glycine, which had been shown to give rise to a folded SFTI-1 analogue. The synthesised analogue was designated as SFTI-G, which exhibited inhibitory activity towards recombinant KLK5 in spectrofluorimetric assay. Further experiments were conducted in this project to evaluate the influences of SFTI-G on the biological functions of KLK5 in keratinocytes.

The inhibition of SFTI-G against exogenous recombinant KLK5 was validated. In addition, the inhibitory activity of SFTI-G towards endogenously overexpressed KLK5 in human keratinocytes was also confirmed. SFTI-G inhibited the unopposed activity of KLK5, further reversing the abnormalities in KLK5-downstream molecules, which are also important proteins involved in skin barrier function. Therefore, inhibition of overactive KLK5 could improve the epidermal barrier function and prevent the development of AD. However, the expression/activity of these downstream molecules of KLK5 in untransduced cells and eGFP-cells was not significantly affected by SFTI-G treatment. It could be theorized that KLK5 was substantially overexpressed in KLK5-cells, where it appeared to be the main target of SFTI-G. Thus SFTI-G bound to active KLK5 predominantly and inhibited the uncontrolled activity of KLK5. Therefore, the abnormalities in KLK5-downstream proteins were reversed after SFTI-G treatment. Nevertheless, SFTI-G can exhibit inhibition towards numerous types of trypsin-like serine proteases, such as other KLKs, cathepsins and matriptases but not only KLK5 (Long et al. 2001; Swedberg et al. 2009; Tan el al. 2013). Thus, SFTI-G could show nonspecific inhibition towards many serine proteases,_and normal function of endogenous serine proteases could be interfered by SFTI-G treatment. Therefore, the therapeutic dosage of potential inhibitor needs to be validated and strictly controlled in future studies.

Although SFTI-G may not be a KLK5-specific inhibitor, the results in this study serve as a useful starting point for the development of more potent and selective inhibitory peptides towards KLK5. In previous reports, an inhibitor that can selectively block the proteolytic activity of KLK4 and KLK7 has been generated through modification of SFTI-1 template structure (Swedberg et al. 2009, 2011; De Veer et al. 2013). These findings raised the possibility that SFTI-1 can be used as a drug scaffold to produce potential analogues, which could specifically inhibit the activity of up-regulated KLK5 and further restore the skin barrier dysfunction in AD patients.

4.2 Conclusion

KLK5 is a key protease expressed in the epidermis and up-regulated KLK5 has been confirmed in the AD skin. Genetic and environmental factors can induce the persistent skin barrier defect in AD, subsequently leading to the consistent up-regulation of KLK5 (Figure 4.1). Our study revealed the importance of overexpressed KLK5 in the development of AD. For example, overexpressed KLK5 triggers the over-degradation of DSG1, further resulting in impaired skin barrier integrity. Furthermore, up-regulated KLK5 could cause the diminished function of PAR2. The consequence of impaired PAR2 function in AD is unclear and still needs to be further investigated. In addition, up-regulated KLK5 may induce disrupted cell growth and aberrant cytokine production in keratinocytes by increasing the levels of p53 and HSP60. Therefore, up-regulation of KLK5 results in the abnormalities in numerous barrier-related proteins, consequently accelerating epidermal barrier defect and promoting the development of AD. Moreover, our results also showed that the impairments caused by up-regulated KLK5 are reversed through inhibition of unopposed KLK5 activity. The results showed reduced degradation of DSG1, restoration of PAR2 activity, decreased expression of p53/HSP60 and decreased production of inflammatory cytokines in KLK5-cells treated with SFTI-G.

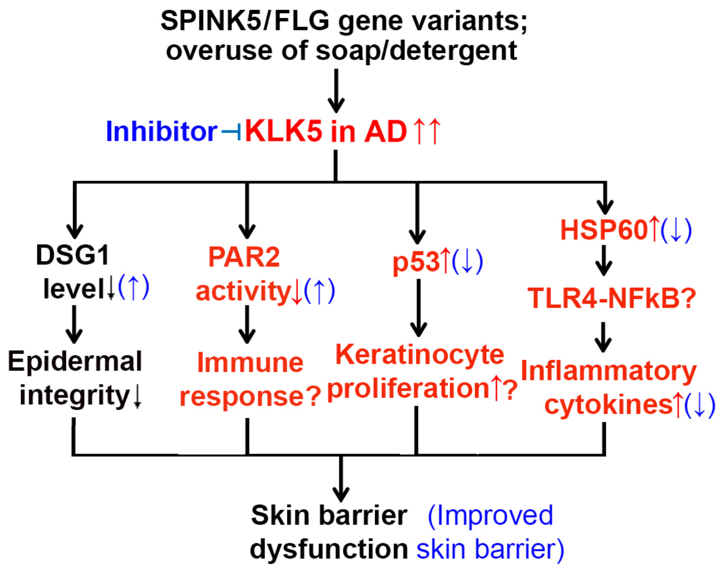

Figure 4.1. Potential roles of up-regulated KLK5 in skin barrier defect in AD.
Genetic factors including polymorphisms in SPINK5 and FLG genes and environmental causes such as overuse of soap can both result in consistent up-regulation of KLK5 in AD. As a result, up-regulated KLK5 results in over-degradation of DSG1 and may induce immune response via PAR2. Overexpressed KLK5 also leads to increased levels of p53 and HSP60, consequently inducing disruption of keratinocyte growth and up-regulation of inflammatory cytokines. These impairments were reversed through inhibition of unopposed KLK5 activity.

As up-regulated proteolytic activity contributes to disturbed epidermal barrier function in AD, restoration of fine-tuned balance between epidermal proteases including KLK5 and their inhibitors represents an important therapeutic intervention for AD. Therefore, application of potential KLK5-specific inhibitor might attenuate unopposed KLK5 activity and counteract the skin barrier dysfunction in AD patients, which is a promising therapeutic approach for AD. SFTI-1 and its analogues possess exceptionally trypsin inhibitory activity against numerous serine proteases including KLKs. Although KLK5 is a one of the major trypsin-like serine proteases expressed in the epidermis, the inhibition of SFTI-1 against KLK5 is not specific. SFTI-1-derevied analogues such as SFTI-G may still be able to inhibit the activity of other trypsin-like serine proteases, thus application of SFTI-G could affect the normal biological functions of other proteases in keratinocytes. It has been revealed that SFTI-1 is a natural bioscaffold that can be modified to become highly specific upon certain targets (Boy et al. 2010; Lesner et al. 2011; Quimbar et al. 2013). In order to improve the selectivity of SFTI-1-derived peptides against KLK5, modification of selected residues will be carried out on the template of SFTI-1, and crystallography will be performed to screen for the potential KLK5-specific inhibitor.

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APPENDIX

1: Relevant genes examined in human EGF signaling PCR array were listed in Table A1. The fold-change and fold regulation values were calculated (Section 2.11).

UniGene	Symbol	Description	Fold-change	Fold up-/down-regulation
Hs.728857	ACTR2	ARP2 actin-related protein 2 homolog (yeast)	0.96	-1.04
Hs.525622	AKT1	V-akt murine thymoma viral oncogene homolog 1	0.54	-1.84
Hs.631535	AKT2	V-akt murine thymoma viral oncogene homolog 2	1.09	1.09
Hs.498292	AKT3	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	1.23	1.23
Hs.446641	ARAF	V-raf murine sarcoma 3611 viral oncogene homolog	0.72	-1.40
Hs.648565	ATF1	Activating transcription factor 1	1.05	1.05
Hs.592510	ATF2	Activating transcription factor 2	1.16	1.16
Hs.370254	BAD	BCL2-associated agonist of cell death	0.94	-1.06
Hs.479747	BCAR1	Breast cancer anti-estrogen resistance 1	0.95	-1.05
Hs.550061	BRAF	V-raf murine sarcoma viral oncogene homolog B1	0.93	-1.08
Hs.141125	CASP3	Caspase 3, apoptosis-related cysteine peptidase	1.08	1.08
Hs.329502	CASP9	Caspase 9, apoptosis-related cysteine peptidase	0.76	-1.35
Hs.504096	CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	1.26	1.26
Hs.523852	CCND1	Cyclin D1	1.03	1.03
Hs.198998	CHUK	Conserved helix-loop-helix ubiquitous kinase	0.78	-1.29
Hs.172928	COL1A1	Collagen, type I, alpha 1	0.82	-1.22
Hs.516646	CREB1	CAMP responsive element binding protein 1	0.86	-1.17
Hs.644056	CSNK2A1	Casein kinase 2, alpha 1 polypeptide	0.98	-1.02
Hs.73527	CSNK2B	Casein kinase 2, beta polypeptide	1.03	1.03
Hs.171695	DUSP1	Dual specificity phosphatase 1	1.15	1.15
Hs.298654	DUSP6	Dual specificity phosphatase 6	0.77	-1.29
Hs.419815	EGF	Epidermal growth factor	0.84	-1.19
Hs.488293	EGFR	Epidermal growth factor receptor	0.69	-1.45
Hs.326035	EGR1	Early growth response 1	0.96	-1.04
Hs.249718	EIF4E	Eukaryotic translation initiation factor 4E	1.14	1.14
Hs.181128	ELK1	ELK1, member of ETS oncogene family	0.73	-1.38
Hs.591160	EPS8	Epidermal growth factor receptor pathway substrate 8	0.75	-1.40
Hs.2007	FASLG	Fas ligand (TNF superfamily, member 6)	0.72	-1.42
Hs.203717	FN1	Fibronectin 1	0.78	-1.29
Hs.728789	FOS	FBJ murine osteosarcoma viral oncogene homolog	0.78	-1.28
Hs.220950	FOXO3	Forkhead box O3	0.86	-1.17
Hs.80720	GAB1	GRB2-associated binding protein 1	0.98	-1.02
Hs.444356	GRB2	Growth factor receptor-bound protein 2	0.75	-1.34
Hs.466828	GSK3A	Glycogen synthase kinase 3 alpha	0.89	-1.13
Hs.445733	GSK3B	Glycogen synthase kinase 3 beta	0.16	-6.11
Hs.799	HBEGF	Heparin-binding EGF-like growth factor	0.73	-1.35
Hs.37003	HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	0.79	-1.27
Hs.597664	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	0.39	-2.59
Hs.89679	IL2	Interleukin 2	0.51	-1.96
Hs.207538	JAK1	Janus kinase 1	0.80	-1.16
Hs.714791	JUN	Jun proto-oncogene	0.93	-1.08
Hs.505033	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0.66	-1.51
Hs.36	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	0.65	-1.53
Hs.145442	MAP2K1	Mitogen-activated protein kinase kinase 1	0.54	-1.87
Hs.514681	MAP2K4	Mitogen-activated protein kinase kinase 4	0.73	-1.44
Hs.531754	MAP2K7	Mitogen-activated protein kinase kinase 7	1.13	1.13
Hs.145605	MAP3K2	Mitogen-activated protein kinase kinase kinase 2	0.89	-1.12
Hs.431850	MAPK1	Mitogen-activated protein kinase 1	1.38	1.38

Hs.125503	MAPK10	Mitogen-activated protein kinase 10	0.38	-2.64
Hs.861	MAPK3	Mitogen-activated protein kinase 3	1.60	1.60
Hs.138211	MAPK8	Mitogen-activated protein kinase 8	1.39	1.39
Hs.484371	MAPK9	Mitogen-activated protein kinase 9	0.88	-1.14
Hs.371594	MKNK1	MAP kinase interacting serine/threonine kinase 1	0.71	-1.42
Hs.2256	MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	1.08	1.08
Hs.529244	NCK2	NCK adaptor protein 2	0.84	-1.19
Hs.632209	NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	0.81	-1.24
Hs.654408	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.79	-1.27
Hs.486502	NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	0.95	-1.05
Hs.467133	NUP62	Nucleoporin 62kDa	0.92	-1.09
Hs.535898	PDGFA	Platelet-derived growth factor alpha polypeptide	0.71	-1.42
Hs.1976	PDGFB	Platelet-derived growth factor beta polypeptide	0.78	-1.29
Hs.74615	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	0.40	-2.50
Hs.459691	PDPK1	3-phosphoinositide dependent protein kinase-1	2.01	2.01
Hs.553498	PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	0.65	-1.55
Hs.132225	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	1.19	1.19
Hs.371344	PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)	0.91	-1.10
Hs.491582	PLAT	Plasminogen activator, tissue	0.82	-1.22
Hs.268177	PLCG1	Phospholipase C, gamma 1	1.53	1.53
Hs.483408	PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme	0.79	-1.27
Hs.531704	PRKCA	Protein kinase C, alpha	0.75	-1.33
Hs.500466	PTEN	Phosphatase and tensin homolog	0.60	-1.67
Hs.159130	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	0.85	-1.17
Hs.190334	RAP1A	RAP1A, member of RAS oncogene family	0.77	-1.30
Hs.664080	RASA1	RAS p21 protein activator (GTPase activating protein) 1	0.72	-1.39
Hs.247077	RHOA	Ras homolog gene family, member A	0.88	-1.16
Hs.510225	RPS6KA5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5	0.87	-1.15
Hs.463642	RPS6KB1	Ribosomal protein S6 kinase, 70kDa, polypeptide 1	1.06	1.06
Hs.433795	SHC1	SHC (Src homology 2 domain containing) transforming protein 1	0.84	-1.19
Hs.642990	STAT1	Signal transducer and activator of transcription 1, 91kDa	0.70	-1.43
Hs.437058	STAT5A	Signal transducer and activator of transcription 5A	0.82	-1.22

Table A1. Expression of EGF-related genes in KLK5-cells and eGFP-cells.
The expression of 82 genes relevant to EGF signaling pathway in KLK5-cells was examined compared to eGFP-cells. The results showed that expression of 19 genes were up-regulated, whereas the expression of 63 genes were down-regulated. Three-fold change was used as the threshold for selection of differential gene expression. The expression of GSK-3β (indicated in red) in KLK5-cells was down-regulated by ~6-fold compared to eGFP-cells.

2: The protein kinases evaluated in human phospho-kinase antibody array were listed in the Table A2. The intensities of the blots were quantified by densitometry (Section 2.13). Normalized protein levels of phospho-kinases in eGFP-cells and KLK5-cells were compared.

Coordinate	Target/control	Mean pixel density	
		eGFP-cells	KLK5-cells
A1,A2	Reference spot	15674	15283
A3,A4	p38-alpha	1441	1494
A5,A6	ERK1/2	1482	1439
A7,A8	JNK1/2/3	1247	1295
A9,A10	GSK3a/b	1124	1119
A13,A14	p53 (S392)	15571	25773
A17,A18	Reference spot	18648	18581
B3,B4	EGFR	1378	1391
B5,B6	MSK1/2	1454	1445
B7,B8	AMPKa1	1386	1361
B9,B10	Akt1/2/3 (S473)	1415	1406
B11,B12	Akt1/2/3 (T308)	1570	1543
B13,B14	p53 (S46)	2593	2659
C1,C2	TOR	1113	1104
C3,C4	CREB	11841	11724
C5,C6	HSP27	1292	1279
C7,C8	AMPKa2	1419	1342
C9,C10	b-cat	28078	28146
C11,C12	p70 S6 kinase	1205	1280
C13,C14	p53 (S15)	1223	1216
C15,C16	c-jun	2497	2555
D1,D2	Src	1319	1302
D3,D4	Lyn	1266	1229
D5,D6	Lck	1345	1375
D7,D8	STAT2	1637	1663
D9,D10	STAT5a	1435	1422
D11,D12	p70 kinase	125	131
D13,D14	RSK1/2/3	1037	1052
D15,D16	Enos	135	129
E1,E2	Fyn	432	411
E3,E4	Yes	536	525
E5,E6	Fgr	603	619
E7,E8	STAT6	1041	1015
E9,E10	STAT5b	1092	1010
E11,E12	STAT3	423	435
E13,E14	p27	1056	1009
E15,E16	PLC-R1	408	399
F1,F2	hck	1145	1156
F3,F4	Chk-2	1063	1039
F5,F6	FAK	425	411
F7,F8	PDGF Rb	524	515
F9,F10	STAT5a/b	506	512
F11,F12	STAT-3	12267	12426
F13,F14	WNK1	16084	15529
F15,F16	PYK2	606	621
G1,G2	Reference spot	16938	16881
G3,G4	PRAS40	16768	16250
G9,G10	Negative control	497	502
G11,G12	HSP60	17909	28985
G17,G18	Negative control	320	374

Table A2. Levels of protein kinases in KLK5-cells and eGFP-cells.
The expression of 43 phospho-kinases and 2 related total proteins in KLK5-cells was examined using human phospho-kinase array compared to eGFP-cells. Mean pixel density of each reference/target was calculated. Increased blot intensities of phospho-p53^{S392} and HSP60 (indicated in red) were detected in KLK5-cells compared to eGFP-cells.

3: The cytokines examined in human cytokine antibody array were listed in the Table A3. The intensities of the blots were quantified by densitometry (Section 2.14). Normalized expression of cytokines in eGFP-cells and KLK5-cells were compared.

Coordinate	**Target/control**	**Mean pixel density**	
		eGFP-cells	**KLK5-cells**
A1,A2	Reference spot	17896	17701
A3,A4	C5/C5a	232	245
A5,A6	CD40 ligand	216	241
A7,A8	G-CSF	308	327
A9,A10	GM-CSF	341	319
A11,A12	GRO-alpha	18958	19110
A13,A14	I-309	327	338
A15,A16	sICAM-1	404	420
A17,A18	IFN-gamma	336	312
A19,A20	Reference spot	16350	16222
B3,B4	IL-1a	188	209
B5,B6	IL-1b	217	232
B7,B8	IL-1ra	15933	15899
B9,B10	IL-2	297	268
B11,B12	IL-4	322	301
B13,B14	IL-5	275	291
B15,B16	IL-6	397	380
B17,B18	IL-8	15922	22598
C3,C4	IL-10	224	8236
C5,C6	IL-12 p70	313	326
C7,C8	IL-13	406	415
C9,C10	IL-16	359	360
C11,C12	IL-17	448	465
C13,C14	IL-17E	385	397
C15,C16	IL-23	535	519
C17,C18	IL-27	449	456
D3,D4	IL-32a	398	386
D5,D6	IP-10	421	409
D7,D8	ITAC	415	406
D9,D10	MCP-1	399	394
D11,D12	MIF	12868	13310
D13,D14	MIP-1a	275	298
D15,D16	MIP-1b	393	415
D17,D18	Serpin E1	18256	18166
E1,E2	Reference spot	17510	17566
E3,E4	RANTES	435	466
E5,E6	SDF-1	449	425
E7,E8	TNF-alpha	398	412
E9,E10	sTERM-1	421	435
E19,E20	Negative control	275	261

Table A3. Production of cytokines in KLK5-cells and eGFP-cells.
The expression of 36 cytokines in KLK5-cells was examined using human cytokine array compared to eGFP-cells. Mean pixel density of each reference/target was calculated. Increased blot intensities of IL-8 and IL-10 (indicated in red) were detected in KLK5-cells compared to eGFP-cells.

4: Protein levels of phospho-kinases in eGFP-cells and KLK5-cells with or without SFTI-G treatment were compared using human phospho-kinase antibody array. The intensities of the blots were quantified by densitometry (Table A4).

Coordinate	Target/control	Mean pixel density			
		eGFP-	eGFP+	KLK5-	KLK+
A1,A2	Reference spot	15674	15538	15283	15502
A3,A4	p38-alpha	1441	1398	1494	1402
A5,A6	ERK1/2	1482	1512	1439	1499
A7,A8	JNK1/2/3	1247	1266	1295	1304
A9,A10	GSK3a/b	1124	1032	1119	1106
A13,A14	p53 (S392)	15571	15639	25773	16022
A17,A18	Reference spot	18648	18301	18581	18992
B3,B4	EGFR	1378	1302	1391	1411
B5,B6	MSK1/2	1454	1414	1445	1477
B7,B8	AMPKa1	1386	1396	1361	1407
B9,B10	Akt1/2/3 (S473)	1415	1399	1406	1425
B11,B12	Akt1/2/3 (T308)	1570	1502	1543	1563
B13,B14	p53 (S46)	2593	2607	2659	2599
C1,C2	TOR	1113	1093	1104	1222
C3,C4	CREB	11841	11792	11724	11821
C5,C6	HSP27	1292	1302	1279	1228
C7,C8	AMPKa2	1419	1444	1342	1393
C9,C10	b-cat	28078	28102	28146	28298
C11,C12	p70 S6 kinase	1205	1199	1280	1203
C13,C14	p53 (S15)	1223	1242	1216	1273
C15,C16	c-jun	2497	2501	2555	2622
D1,D2	Src	1319	1359	1302	1314
D3,D4	Lyn	1266	1251	1229	1325
D5,D6	Lck	1345	1422	1375	1408
D7,D8	STAT2	1637	1625	1663	1709
D9,D10	STAT5a	1435	1398	1422	1409
D11,D12	p70 kinase	125	111	131	150
D13,D14	RSK1/2/3	1037	1112	1052	1098
D15,D16	Enos	135	123	129	161
E1,E2	Fyn	432	425	411	509
E3,E4	Yes	536	512	525	587
E5,E6	Fgr	603	699	619	704
E7,E8	STAT6	1041	998	1015	1021
E9,E10	STAT5b	1092	1142	1010	1265
E11,E12	STAT3	423	399	435	502
E13,E14	p27	1056	1110	1009	1099
E15,E16	PLC-R1	408	415	399	325
F1,F2	hck	1145	1099	1156	1251
F3,F4	Chk-2	1063	1113	1039	1212
F5,F6	FAK	425	506	411	465
F7,F8	PDGF Rb	524	499	515	532
F9,F10	STAT5a/b	506	586	512	604
F11,F12	STAT-3	12267	12315	12426	12601
F13,F14	WNK1	16084	15456	15529	16123
F15,F16	PYK2	606	666	621	709
G1,G2	Reference spot	16938	16910	16881	16823
G3,G4	PRAS40	16768	16617	16250	16438
G9,G10	Negative control	497	520	502	616
G11,G12	HSP60	17909	17615	28985	18821
G17,G18	Negative control	320	298	374	333

Table A4. Levels of protein kinases in cells with or without SFTI-G treatment.
The expression of 43 phospho-kinases and 2 related total proteins in KLK5-cells treated with SFTI-G was examined using human phospho-kinase array compared to un-treated cells and eGFP-cells. Mean pixel density of each reference/target was calculated. Decreased blot intensities of phospho-p53^{S392} and HSP60 (indicated in red) were detected in KLK5-cells treated with SFTI-G but not in untreated cells.

5: The profiles of cytokines expression in eGFP-cells and KLK5-cells with or without SFTI-G treatment were compared using human cytokine antibody array. The intensities of the blots were quantified by densitometry (Table A5).

Coordinate	Target/control	Mean pixel density			
		eGFP-	**eGFP+**	**KLK5-**	**KLK+**
A1,A2	Reference spot	17896	17221	17701	17193
A3,A4	C5/C5a	232	294	245	302
A5,A6	CD40 ligand	216	259	241	226
A7,A8	G-CSF	308	299	327	302
A9,A10	GM-CSF	341	367	319	298
A11,A12	GRO-alpha	18958	19020	19110	19212
A13,A14	I-309	327	381	338	306
A15,A16	sICAM-1	404	394	420	441
A17,A18	IFN-gamma	336	385	312	397
A19,A20	Reference spot	16350	16361	16222	16119
B3,B4	IL-1a	188	226	209	198
B5,B6	IL-1b	217	275	232	202
B7,B8	IL-1ra	15933	15659	15899	16021
B9,B10	IL-2	297	310	268	289
B11,B12	IL-4	322	338	301	297
B13,B14	IL-5	275	311	291	298
B15,B16	IL-6	397	344	380	393
B17,B18	IL-8	15922	15273	22598	16276
C3,C4	IL-10	224	243	8236	268
C5,C6	IL-12 p70	313	289	326	301
C7,C8	IL-13	406	484	415	461
C9,C10	IL-16	359	331	360	314
C11,C12	IL-17	448	429	465	438
C13,C14	IL-17E	385	375	397	408
C15,C16	IL-23	535	521	519	569
C17,C18	IL-27	449	425	456	406
D3,D4	IL-32a	398	331	386	350
D5,D6	IP-10	421	452	409	398
D7,D8	ITAC	415	429	406	466
D9,D10	MCP-1	399	411	394	429
D11,D12	MIF	12868	12525	13310	12806
D13,D14	MIP-1a	275	310	298	322
D15,D16	MIP-1b	393	404	415	438
D17,D18	Serpin E1	18256	18010	18166	18125
E1,E2	Reference spot	17510	17725	17566	17621
E3,E4	RANTES	435	509	466	428
E5,E6	SDF-1	449	399	425	462
E7,E8	TNF-alpha	398	439	412	402
E9,E10	sTERM-1	421	461	435	489
E19,E20	Negative control	275	211	261	293

Table A5. Production of cytokines in cells with or without SFTI-G treatment.
The expression of 36 cytokines in KLK5-cells treated with SFTI-G was examined using human cytokine antibody array compared to un-treated cells and eGFP-cells. Mean pixel density of each reference/target was calculated. Reduced blot intensities of IL-8 and IL-10 (indicated in red) were detected in KLK5-cells treated with SFTI-G but not in untreated cells.